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NOVEL APPROACHES TO MUCOLYSIS TO IMPROVE AIRWAY MUCUS
CLEARANCE IN CYSTIC FIBROSIS

by

BONNIE DASGUPTA



A thesis submitted to the Faculty of Graduate Studies and Research in partial
fulfillment of the requirements for the degree of MASTER OF SCIENCE

in

EXPERIMENTAL MEDICINE

Department of Medicine

Edmonton, Alberta

Spring 1995



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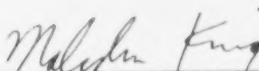
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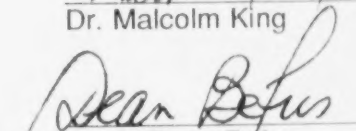
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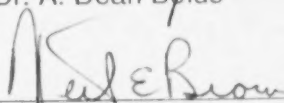
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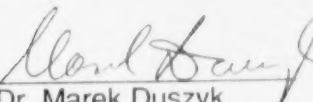
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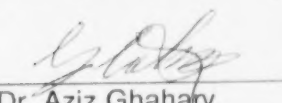
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Dr. Malcolm King


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Dr. Aziz Ghahary

March 21, 1995

To my parents, Mrinal and Shibani,
and my sister Tina.

ABSTRACT

In cystic fibrosis (CF) lung disease, the viscous character of the mucous gel is dependent upon a number of forms of bonding. The objective of this thesis was to examine novel approaches to mucolysis (disrupting the mucous gel), based on the molecular nature of crosslinking and bonding in mucin gels, to facilitate mucus clearance in CF patients.

Experiments were carried out *in vitro* using sputum collected from CF patients. The individual and combined effects of five representative mucotropic modalities (Oscillation, rhDNase, Gelsolin, Nacystelyn, and Hypertonic Saline) were examined, using rhDNase (Pulmozyme®), a licensed mucolytic, as the basis for comparison with the four other mucotropic agents. The effects of mucolysis on airway mucus was determined by measuring: 1) viscoelasticity, 2) spinnability, and 3) clearability.

Significant reductions in spinnability of CF sputum were demonstrated with each mucotropic agent; combined treatments of mucotropic agents were generally more efficacious at reducing spinnability than individual treatments. These findings suggest that synergy between mucotropic agents deserves consideration in treating disorders of mucus clearance in CF patients.

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Chapter I
INTRODUCTION

Cystic fibrosis [CF] is a life-threatening genetic disorder that affects children and young adults (1). CF is inherited in an autosomal recessive fashion: heterozygotes carrying one normal CF allele and one mutant allele are asymptomatic and classified as carriers. On the other hand, a child of two carriers has a one in four chance of inheriting a genetic mutation from each parent and of being affected. It has been estimated that 3.3% of Caucasian people in the United States of America (more than 7 million people) are symptom-free carriers of the CF gene (2). The frequency of CF varies among sexes and among ethnic groups. The National CF Patient Registry of the United States of America reported 17,857 CF patients in 1990 of which 53.8% were males. Patients can be divided by race as follows: 95.2% were Caucasian, 3.1% were African American, 0.2% were Asian or Pacific Islander, 0.2% were American Indian, Aleut, or Eskimo, and the ethnic origins of 1.3% were unknown (3). The Canadian CF Patient Registry of the Canadian CF Foundation reported 2,860 CF patients in 1992.

CF was discovered early in the 20th century. In 1938, Anderson first introduced the term "cystic fibrosis of the pancreas" to acknowledge the destruction of the pancreatic exocrine function as a result of this disease (4). A few years later, in 1953, Di Sant'Agnese reported that sweat from CF children demonstrated excessive salt loss (5). Di Sant'Agnese's finding soon led to the measurements of sodium and chloride in sweat as a diagnostic standard for CF disease (6). Approximately 10 years ago, the salt transport abnormality was further defined by Knowles *et al.* who reported a similar abnormality in salt transport in the respiratory epithelium (7-8). In 1989, researchers in Toronto discovered the CF gene, which comprises 27 exons and 250 kilobases, and resides on chromosome 7 (9-11). The CF gene codes for a protein known as the cystic fibrosis transmembrane conductance regulator (CFTR). The CFTR

protein acts a chloride channel in the apical membrane; hence its absence is able to account for the abnormalities in electrolyte and water content in the airway surface fluid (ASF) in CF lung disease.

Due to the development of specialized CF clinics and improved clinical management, CF patients have a longer life expectancy. In fact, the survival rate quadrupled between 1969 and 1992. According to the Canadian CF Patient Registry, 56 CF patients died in Canada during 1992. During 1990 in the United States of America, there were a total of 411 deaths with a crude mortality rate of 2.3 per 100 (3). The causes of these deaths were broken down as follows: cardiorespiratory [78.1%], hepatic [3.4%], trauma [1.0%], and suicide [0.7%]. The lowest mortality rate was observed in those aged 2 to 10 years [1.1%] and the highest mortality rate was observed in those aged more than 41 years [5.2%] (3). Data from 1969, 1972, and 1978 were compared revealing a significant shift in age distribution of CF patients. A fourfold increase was shown in the proportion of adult CF patients between 1969 [8%] and 1990 [33%] (3).

The most important clinical manifestation of CF is chronic progressive lung disease which, despite aggressive antibiotic therapy for recurrent infections, and chest physiotherapy, remains the principal cause of disability and death in CF patients. Respiratory dysfunction in CF is predominantly due to the thick and purulent airway secretions (known as sputum). The viscous sputum is difficult to expectorate, obstructing the airways of the CF patient and contributing to their reduced lung volumes and expiratory flow rates.

Normally, airway mucus functions as a protective layer lining the conducting airways of the respiratory tract. Airway mucus represents the first line of defense against inhaled particles and pathogens. Primarily, mucus is a biological gel, 90-95% of which is composed of water and 1-2% of mucous glycoproteins. Under normal conditions, less than 1% of airway mucus is

composed of proteins, lipids, and ions of low molecular weight. In patients suffering from CF, between 3 to 4% of airway mucus is composed of the latter. Infected mucus also contains DNA, F-actin, and proteolytic enzymes released from bacteria and host leukocytes.

The three-dimensional structure of the mucous gel is dependent on numerous types of molecular bonding and crosslinkages: 1) *covalent bonds*, 2) *ionic bonds*, 3) *hydrogen bonds [H-bonds]* 4) *van der Waals forces*, and 5) *physical entanglements*. Additional crosslinking is derived from extra macromolecules such as DNA, F-actin, and certain proteins. Water, one of the major components of mucus, is held in various states within the mucous gel. Water is associated with fixed ions, H-bonds, and more loosely with the entangled gel network.

As mentioned before, normal, free-flowing airway mucus becomes viscous and purulent in patients suffering from CF lung disease. Therefore, current approaches in the treatment of CF lung disease include strategies for changing the physical properties of pulmonary secretions to improve lung clearance in CF patients. The change in the physical property of the CF sputum from a nonflowing gel to a flowing liquid allows the CF patient to expectorate larger quantities of sputum, improving the patient's mucociliary clearance rate. This change in the physical property of sputum can be carried out through the disruption of the mucous gel, a process known as *mucolysis*.

Mucolysis can be achieved directly or indirectly. Treating pre-formed mucus with either physical (such as high frequency oscillation) or biochemical agents (such as recombinant human deoxyribonuclease I [rhDNase] or Nacystelyn [NAL]) can be considered "direct mucolysis". Therapy aimed at improving the electrolyte and water content of ASF, using amiloride and/or uridine triphosphate (UTP) (12), may be considered a form of "indirect

mucolysis". Other forms of therapy for CF lung disease range from lung transplantation to gene therapy (13,14).

To date, the relationship between the individual and combined effects of mucolysis and clinical outcomes has not yet been determined. Therefore, the objective of this thesis was to examine novel approaches to mucolysis to improve airway mucus clearance in CF. If successful results are obtained, then combined treatment with mucolytic agents on CF patients may be able to improve their quality of life and increase the cost-effectiveness of treatment.

The approaches to mucolysis was based on the molecular nature of crosslinking and bonding in mucin gels. A comprehensive review of the structure and biochemistry of mucus is presented in chapter two of this thesis. This will help to comprehend the different types of mucolysis in terms of their mechanism of action on the structure and biochemistry of the mucous gel. As an example, the chemical modification of the gel network by: (i) S-S bonds which can be broken through reducing agents, changing the ionic strength, or reducing the degree of extra crosslinking due to DNA, or (ii) proteins associated with infection and cell degradation. Another example is to observe the physical disruption of the mucous gel by high frequency oscillation.

The effects of various approaches to mucolysis were tested by studying the different physical properties of airway mucus. They were determined by measuring: 1) *viscosity and elasticity* [the basic parameters describing the liquid-like and solid-like properties of the mucous gel], both of these parameters were measured by means of a magnetic microrheometer; 2) *spinnability and surface properties*. Spinnability, the capacity of mucus to form threads under traction, correlates with mucous viscoelasticity and clearability and was measured using a filancemeter. Surface properties are important to determine how mucus adheres to epithelial lining and how it interacts with airflow.

The experiments were carried out *in vitro* using sputum obtained from CF patients. The individual and combined effects of five different mucotropic modalities were tested: (1) rhDNase, (2) Oscillation, (3) Gelsolin, (4) NAL, and (5) Hypertonic Saline. The basis for comparison with the four other mucotropic agents was rhDNase (Pulmozyme®), a licensed mucolytic agent. For standardization, tracheal mucus, collected from anaesthetized healthy dogs, were treated with rhDNase to observe any differences in viscoelasticity of healthy mucus.

Within this thesis, chapter three deals with the individual and combined *in vitro* effects of physical and biochemical mucotropic agents, i.e. rhDNase and oscillation. Chapters four and five will deal with the synergistic effects of mucotropic agents *in vitro*. Chapter four concerns rhDNase and Gelsolin and chapter five concerns rhDNase and NAL. Chapter six deals with the additive effects of rhDNase and Hypertonic Saline. Sputum samples were obtained from CF patients from different centers (Edmonton, Winnipeg, and Sydney, Australia). The common goal of these studies was to determine which mucotropic agents, single or combined with another, have the best chance of improving airway mucus clearance in CF patients.

The treatment of CF lung disease is progressing slowly but steadily. Independent results on high frequency oscillation (15) and rhDNase (16,17) show encouraging results. By combining mucotropic modalities (such as rhDNase and oscillation), novel approaches to mucolysis were examined *in vitro* in CF patients. It is hoped that the consequences of this research may be used for development of better therapies which will improve cost-effectiveness of treatment, and improve the quality of life of people suffering from CF.

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Chapter II

MOLECULAR BASIS FOR MUCOLYTIC THERAPY

INTRODUCTION

Airway mucus is the basic mode of protection in the lungs. It traps inhaled dust particles and bacteria and removes them from the airway by means of either ciliary clearance or coughing. Airway mucus is a complex viscoelastic gel. It is composed mainly of water (90-95%) and mucins [or mucous glycoproteins] (1-2%). Under normal conditions, less than 1% of airway mucus is composed of proteins, lipids, and low molecular weight ions. In patients suffering from cystic fibrosis [CF], 3 to 4% of airway mucus is composed of the latter (1).

The viscoelastic properties of airway mucus can be attributed predominantly to its mucin content (2). Mucins are described as having a large size, polydisperse, and an unusual molecular structure. Airway mucins are synthesized and secreted by a number of specialized cells [e.g. mucous and serous cells of the submucosal glands, and goblet and Clara cells of the surface epithelium]. Mucins have particular characteristics that distinguish them from other components of mucus, as well as from most other glycoproteins.

Mucins have a complex structure. They are composed of a polypeptide core, which is rich in serine, threonine and proline, and numerous oligosaccharide side chains. These side chains are connected to the polypeptide core through O-glycosidic linkages via N-acetylgalactosamine (3). The most prominent feature of mucins is their large size; the molecular weight of mucins range between three and seven million daltons. Although they have been described as highly expanded molecules, mucins are reported to behave as random coils (4).

Polydispersity is another feature demonstrated by mucins. Various studies have shown that mucins differ in their peptide length (4,5). Investigators report that mucin peptide units are linked head-to-tail. Tandem repeats of amino

acid sequences seem to be typical, as was demonstrated by the amino acid sequence data obtained from cDNAs of several mucins (6-10). As well as peptide variability, mucins are heterodisperse with regard to their oligosaccharide composition and length (11).

The purpose of this paper is to look at several approaches to mucolysis [disruption of the mucous gel] based on the molecular nature of crosslinking and bonding in mucin gels. For background purposes, a comprehensive review of the structure and biochemistry of mucus will be presented in the first five sections. This will help to comprehend the different types of mucolysis in terms of their mechanism of action on the structure and biochemistry of the mucous gel. As examples, we will describe the chemical modification of the gel network by S-S reducing agents, changing ionic strength, and/or reducing the degree of extra crosslinking due to DNA or proteins associated with infection and cell degradation. Also, we will demonstrate the physical disruption of the mucous gel by high frequency oscillation, and how the addition of surfactants modifies the surface and bulk-phase rheology. Following the structure and biochemistry of mucus, the subsequent sections will deal with the different physical and biochemical approaches to mucolysis that act upon the mucous gel. Seven representative mucotropic modalities (N-acetylcysteine, Urea, Hypertonic Saline, rhDNase, Gelsolin, Oscillation, and Surfactants) will be presented, all of which act upon different components of the mucous gel. The paper will conclude by looking at mucolytic synergism involving these various agents.

FUNCTIONAL ASPECTS OF AIRWAY MUCUS

Airway mucus has a number of functions within the human body. Though harmful in excessive quantities, airway mucus is generally beneficial in its effects on humans. The primary advantageous feature of airway mucus is that it

plays a protective role by maintaining a physical barrier that guards against harmful inhalants (12-15). Invasion of bacteria and foreign particles is prevented by direct interaction of mucin molecules with these foreign bodies (16,17), this being a crucial stage in the pathogenesis of the infection. Occasionally, airway mucus acts as a mechanical buffer, shielding the underlying tissue from mechanical damage. At this time, airway mucus protects the tissue from molecular invasion through blockage and entrapment of the molecules. By penetrating into the mucous layer, the individual cilium transfers momentum and then disengages the mucus in order to execute a return stroke through the non-viscoelastic periciliary fluid layer (18,19).

In contrast to its benefits, large amounts of airway mucus may have a negative function. People suffering from diseases characterized by mucous hypersecretion [e.g. chronic bronchitis, CF] have a large amount of mucus obstructing their airways, particularly the small airways causing dyspnea. Respiratory dysfunction in CF patients is predominantly due to the thick and viscous airway secretions. These thick and purulent secretions are difficult to expectorate, thus obstructing the airways of the patient and contributing to their reduced lung volumes and expiratory flow rates.

SOL & GEL PHASE OF MUCUS

The airways of the respiratory tract are lined with a double layer of mucus, a surface [superficial] gel layer and an underlying sol layer. The gel layer is produced from the mucous glands and the goblet cells in the airways. However, the origins of the sol layer remain controversial. Lucas and Douglas (20) reported that the sol layer is produced either from the serous cells of the mucous glands or is a transudate from the cells lining the airways. Kilburn (21) suggested that the sol phase is generated by alveolar type II cells and by Clara

cells. However, Negus (22) argued that both the sol and gel phases have the same origins; the separation into two distinct layers is due to thixotropy [a state in which the pressure from the cilia beating causes rearrangement of the gel layer]. The gel and sol layers are useful to describe both mucous transport and the differences in the distribution and concentration of mucous glycoproteins and serum component in the sol and gel layers.

The notion of gel and sol phase makes it simple to understand mucous transport, especially the high velocity of transport compared with the frequency of beat and length of cilia. In 1961, Hilding (23) demonstrated the rate of mucociliary clearance in animals to be 15 mm/min. It was explained that this high velocity was due to the gel layer which interacted with the ciliary tips during the effective phase of the beat, and perhaps during the peak velocity of that phase. During the recovery phase, the cilium interact only with the sol layer, and each cilia is a bit ahead in the phase of the cycle from the one immediately behind it. In other words, Hilding demonstrated the reverse direction which is taken by the waves of the recovery stroke in the gel phase. However, Sleight *et al.* suggests that the sol layer shows little net movement (24).

Differences in the distribution and concentration of mucous glycoproteins and serum component in the sol and gel layers are determined by high speed centrifugation of the bronchial mucus into two distinct phases: sol phase and gel phase. The sol phase is composed of lipids, proteins, mucous glycoproteins, and DNA in solution, while the gel phase contains the same constituents as the sol phase but is found in a gel structure. Upon centrifugation, non-infected sputum exhibits two distinct patterns of separation: a bronchorrhea or "sol" type phase where the sol represents 85% or more of the total bronchial secretion and a "gel" type phase where the percentage varies between 10 and 15% (25). Non-infected sputum from asthmatics can separate either as a "gel" or a "sol"

type phase. Infected sputum from chronic bronchitis and CF patients demonstrates a vast range of reactions. Generally the secretion separates into the two phases [similar to the non-infected sputum], but some types of secretions separate either as sol or gel transudate.

Mucous glycoproteins are present in both the gel and the sol phases; however, a higher concentration is found in the gel phase. In the sol phase, the mucous glycoprotein is present in a soluble form. This distribution of mucous glycoprotein has been reported in all cases of pulmonary disease, both infected and non-infected. Nevertheless, Bhaskar and Reid (26) reported an exception to this rule: in two cases of asthma [one intrinsic and the other extrinsic] no mucous glycoprotein was detected in the sol phase.

Serum transudate proteins, such as albumin, transferrin, and IgG, are found in both the sol and gel phases with a higher concentration being found in the sol phase rather than the gel phase. This indicates mobility of these proteins and suggests a weak association with mucous glycoproteins (2).

BIOCHEMICAL MAKE-UP OF MUCUS

(1) MUCINS

Airway mucins are composed of carbohydrates [75-85%], sulfates [1-8%], and peptides [10-20%] (11). They consist of a linear peptide core with oligosaccharide side chains. N-acetylgalactosamine covalently links the mucin carbohydrate chains with the hydroxyl group of serine (27). This linkage may be revealed by treatment of purified glycoproteins with mild alkaline [0.05 M], which permits the removal of oligosaccharide chains from the peptide through a β elimination reaction (27).

The carbohydrate component of airway mucins is made up of five different sugars: D-N-acetylgalactosamine, D-N-acetylglucosamine, D-

galactose, neuraminic acid [sialic acid], and L-fucose. Mucin carbohydrate structures are quite complex. They can be divided into to three distinct groups: (1) cores, (2) backbones, and (3) peripherals (28-37). There are four core structures which are found next to the peptide core: (1) Gal β 1-3GalNAc α -O-Ser/Thr, (2) Gal β 1-3(GlcNAc β 1-6)-GalNAc α -O-Ser/Thr, (3) GlcNAc β 1-3GalNAc α -O-Ser/Thr, and (4) GlcNAc- β 1-3(GlcN β 1-6)GalNAc α -O-Ser/Thr (11). The mucin carbohydrate structure also contains four backbone structures: (1) type 1, Gal β 1-3GlcNAc, (2) type 2, Gal β 1-4GlcNAc, (3) i antigen, GlcNAc β 1-3Gal, and (4) I antigen, GlcNAc β 1-3(GlcNAc β 1-6)Gal (11). The peripheral component of the mucin carbohydrate structure is more diverse. For example, sialic acid can link to C-3 or C-6 of galactose (38-40).

Synthesis of mucin oligosaccharides depend on a number of factors, including availability of donor nucleotide sugars and the pH in the Golgi lumen (41). Oligosaccharide size is variable and the chains can be neutral or acidic, depending on the presence or absence of sialic acid and/or sulfate ester. Mucin oligosaccharide structures also vary in anomeric configuration and linkages. For instance, galactose can link to an adjacent sugar as either an α - or β -anomer (11).

Unfortunately, the carbohydrate composition should not be used to measure the purity of mucins as the carbohydrate content changes from mucin to mucin [microheterogeneity]. However, the absence of sugars, which are normally not found in purified mucins, is an adequate measure of purity. For example, impurity of mucins may be exhibited by the presence of mannose [a typical component of proteoglycans] (7,8).

Seventy to eighty percent of the peptides within the mucins are composed of glycine, proline, alanine, glutamic acid, serine, and threonine (11). Together the two amino acids, serine and threonine, compose 30 to 50 mol % of

the total peptide content. Serine and threonine, which yield the o-glycosidic linkages, are present in much larger concentrations in mucins than other polypeptide cores. For instance, human tracheobronchial mucin has higher concentrations of threonine than serine, whereas several other mucins have equal or higher concentrations of serine than threonine (4).

The composition of amino acids of airway mucins varies from species to species. The degree of variability of the amino acid composition of airway mucins is not known as the compositional data cannot predict the peptide structure. However, amino acid sequences deduced from cloned human airway mucin cDNAs give more information about the mucin peptide structure (42). The cloned human cDNAs indicate that hydroxyamino acids do not distribute evenly along the peptide chain. It so happens that the bulk of these amino acids is found in a tandem repeat domain of the molecule (9,42). The oligopeptide tandem repeats and their size differs among the airway mucin genes (9,42-43).

(II) PROTEINS & LIPIDS

Proteins in mucus are mainly derived from serum transudate and play a major role in the host defence mechanisms of the airway. By interaction with bronchial glycoprotein, proteins also contribute to the viscoelastic properties of airway secretions. Air space is normally separated from vascular space by a filtration barrier that excludes larger molecules, but allows movement of smaller molecules through endothelial and epithelial pores (44). With airway inflammation, the barrier begins to leak; therefore, larger concentrations of serum proteins are apparent in inflamed airways [e.g. bronchitic airways] in comparison to healthy airways (45).

Proteins are synthesized from different types of sources. A certain number of proteins is synthesized and secreted from epithelial cells, while

another source is the inflammatory cells which are secreted or released from cell disintegration [e.g. lysozyme, IgA]. Nonetheless, serum proteins [e.g. IgG, IgA, and lysozyme] have significant roles in airway secretions. The immunoglobulins have protective functions and the antiproteases inactivate proteolytic enzymes which are released by inflammatory cells. Serum proteins may also help to determine the rheological properties of airway mucus.

Lipids and proteins are found in approximately equal concentrations in airway mucus. Lipids are derived from a number of different sources, such as alveolar surfactants, which originate from the cells of the conducting airways, and membranes of disrupted cells (46). The largest concentration of lipids is found in viscous airway secretions [e.g. CF patients]. The lipid content in CF sputum is 3.1% on a wet weight basis (46). In comparison, sputum from laryngectomized patients has a 1% lipid content on a wet weight basis (47). Lipids, which are found in airway secretions, are composed of neutral lipids, phospholipids, and glycolipids. Mucus from healthy dogs and sputum from healthy patients exhibited moderate amounts of neutral lipids and smaller amounts of phospholipids (15). Bhaskar *et al.* (14) compared the lipid content *in vitro* and *in vivo* and found the following results: glycolipid content increased following chronic SO₂ exposure in dogs. Yet they claimed that glycolipids found in CF patients are products of undefined pathological processes (47).

THE CROSS-LINK

The thick and elastic airway mucus secretions illustrate characteristics of a gel. A gel is described as a solution where the macromolecules are all linked together [at least one cross link per chain] to produce very large macroscopic aggregates (48). The molecular nature of crosslinking of airway mucus is dependent upon a number of different types of bonds. Therefore, in order to

reduce the viscosity and elasticity of the tenacious mucus, the crosslinking and bonding within the mucous gel must be broken.

A normal mucous gel is composed of numerous bonds (Fig. II-1) including *covalent bonds* [such as disulfide bonds], *hydrogen bonds* [which bind two hydroxyl groups], *ionic bonds* [which often bind sulfated sugar units to amino groups], *van der Waals forces* [weak, attractive forces which contribute to interdigitation between neighbouring molecules], and *physical entanglements* [which refer to mechanical catching between polypeptide chains] (49). In short, all of these bonds contribute to crosslinkages within the mucous gel. In normal, noninfected mucus, the majority of the bonding is due to mucins [mucous glycoproteins]. Additional factors related to cross-linking within the mucous gel will modify the physical properties of the gel; high water content will lower viscosity (50) while the addition of deoxyribonucleic acid [DNA] (51) or serum albumin (52) will raise it. However, in mucus produced by chronic irritation, such as smoking, the mucin macromolecules within the mucous gel develop an excess of fixed negative charges, as evidenced by the histochemical shift to acidic mucin (53). This excess of negative charges develops a net repulsion making the mucous gel highly acidic. In chronic airway infection, such as CF, in addition to the mucin macromolecules, the mucous gel also contains DNA and F-actin [both of which are released from dying cells and bacteria] as well as bacterial alginate. These affix additional crosslinkages to the mucous network. The DNA from the host leukocytes has a very high molecular weight and is quite stiff, thereby forming a rigid network. In CF, DNA concentrations are often sufficient to make the parallel DNA network stronger than the mucin matrix.

GENERAL APPROACHES TO MUCOLYSIS & MUCOKINESIS

To change the physical properties of the viscous and rigid mucous gel, the direct strategy is Mucolysis, but two indirect strategies may also be involved, namely Secretory Inhibition and Mucokinesis. *Mucolysis* refers to the disruption of the mucous gel, generally by altering the degree of crosslinking or the interactions between the macromolecules that form the gel. Normally it is desirable to reduce the crosslinking in the mucous gel in order to improve clearance, but occasionally, the mucus will be too thin for effective transport (54,55); hence, increasing the crosslinking of the mucus by a *Mucospissic* agent could be appropriate. *Mucolytics* and *mucospissics* are known collectively as *mucotropic* agents.

Secretory inhibition involves reducing mucous hypersecretion by using pharmacological agents. This can be accomplished by employing anticholinergic agents, for example atropine (56). An opposite approach to secretory inhibition is to promote mucous secretion by the use of secretagogues [e.g. *expectorants*]. *Secretagogues* promote fresh secretions within the glycoprotein gel. These new secretions displace the old bacteria laden layer, clearing the airways of some of its burden of pathogens. Changing the secretion rate does not necessarily involve altering the rheology of the mucus, but it will likely do so if macromolecular secretion and water transport are affected in a differential fashion.

The second strategy is known as *Mucokinesis*, where the objective is to physically move the mucus from smaller to larger airways from where it can be eliminated by cough clearance. Mucokinesis does not necessarily imply mucolysis, especially if the mucokinesis occurs primarily through stimulation of the cilia, or through airflow or gravity mechanisms [the two primary mechanisms used to move mucus]. However, agents that alter cilia beating often also alter

mucous rheology. For instance, physiotherapy methods, such as high frequency chest compression, involve airflow regimes that have the potential to alter mucous rheology as well as stimulate clearance.

Mucus can be considered as a viscoelastic fluid, since it exhibits both liquid-like (viscous) and solid-like (elastic) properties. *Viscosity* is the resistance to flow and represents the capacity of a material to absorb energy as it moves. *Elasticity* is the capacity of a material to store energy used to move or deform it. The relative proportions of elasticity and viscosity are as important in describing how a material such as mucus behaves when it is subjected to external forces as are the absolute values of either parameter by itself.

The effects of different approaches to mucolysis can be tested by studying the different physical properties of airway mucus. This can be determined by measuring: 1) *viscosity and elasticity* [the basic parameters describing the liquid-like and solid-like properties of the mucous gel]. Both of these parameters is measured by means of a magnetic microrheometer; 2) *spinnability and surface properties*. Spinnability – the capacity of mucus to form threads under traction – correlates with mucous viscoelasticity and clearability. Spinnability is measured using a filancemeter. Surface properties are important to determine how mucus adheres to epithelial lining and how it interacts with airflow; 3) the effect on *clearance of airway mucus* by the two major relevant mechanisms: *ciliary action and airflow*.

INTRODUCTION TO MUCOTROPIC AGENTS

The aforementioned are only general approaches to mucolysis. In order to attain the maximal effects of mucolysis, it is essential to look at the complex mucous gel. As mentioned before, airway mucus is composed of a number of different types of bonds [e.g. *covalent bonds, ionic bonds, hydrogen bonds etc.*]

(Fig. II-1). Breakage or reduction of the bonds within the mucous gel can be achieved through disruption of the mucous gel, which is known as Mucolysis. Now mucolysis can be achieved through pharmacological agents [also referred to as Mucotropic agents], either through physical interaction, such as High Frequency Oscillation, or by biochemical agents, such as rhDNase or N-acetylcysteine. Thus, by breaking the bonds, mucolysis will result in reducing the viscoelasticity of the mucous gel, to a certain extent, enabling the patient to achieve optimal clearance of mucus. Consequently, this will improve the patient's lung volumes and expiratory flow rates.

The array of mucotropic agents which will be described in the following sections are within a group entitled primary agents which directly affect either the production of sputum or change the characteristics of the sputum. There are many different types of primary mucotropic agents. In this thesis, seven representative examples are looked at: N-acetylcysteine, Urea, Hypertonic Saline, rhDNase, Gelsolin, Oscillation, and Surfactants. Each of these mucotropic agents are meant to disrupt or modify a separate bond within the mucous gel (Table II-1).

N-ACETYLCYSTEINE

N-acetylcysteine (NAC) has been widely used as a mucolytic agent because of its excellent characteristics. It reduces the viscosity of purulent sputum in both CF and chronic bronchitis patients, and enhances the removal of pulmonary secretions by ciliary action or cough, hence reducing the need for repeated aspiration (57,58). Among the sulfhydryl-containing compounds, NAC has been recognized for demonstrating the greatest mucolytic effects in humans with numerous pulmonary disorders (59). Studies which used oral NAC to reduce acute bronchial exacerbations report NAC to behave as a free radical

scavenger in the lung, thereby employing protective effects on bronchial secretions (60). NAC breaks down the disulfide bonds of the mucous gel (Fig. II-1), reducing its viscosity and enhancing expectoration.

NAC is a derivative of cysteine and is a thiol reducing agent. The chemical structure of disulfide-containing proteins and peptides which are connected by disulfide linkages are altered by thiol compounds (61). Physical changes in the bronchial glycoprotein are the result of thiol reduction; they are associated with reduction in molecular size, sedimentation coefficient, and viscosity (25). NAC reduces the disulfide bond (S-S) to a sulfhydryl bond (-SH). A free sulfhydryl group is successful in reducing the elasticity and viscosity of mucus (62). Previous studies have shown a correlation between the reduction in mucous viscosity and the reduction of disulfide bonds (59). These mucolytic properties of NAC were tested and confirmed [using a magnetic microrheometer] on the tracheal mucus of dogs (63).

In vitro studies of NAC on the viscosity of tracheobronchial secretions report a dose-related activity: the greater the concentration of NAC administered the greater the decrease in viscosity (59,64). In terms of purulent and nonpurulent sputum, NAC demonstrated similar effects on both types of sputum. Other *in vitro* studies reported greater mucolytic activity of NAC in solutions with a pH over a range of 5.5 to 8.0 (65).

Short-term *in vivo* studies of NAC [for a period of two weeks] on adults and children suffering from acute bronchopulmonary diseases reported that the sole use of NAC or the use of NAC in combination with antibiotics decreased sputum consistency, eased expectoration, and markedly reduced cough and thoracic physical signs (66,67). In chronic bronchitis patients, comparing the administration of a placebo to administration of NAC for a period of four weeks, NAC proved to be the better of the two treatments in reducing sputum viscosity

and cough severity (68). However, parameters such as difficulty of expectoration, and the severity of cough had the same conventional levels of statistical significance for the two treatments [$0.1 > p > 0.05$] (69). In contrast to short-term *in vivo* studies, long-term *in vivo* studies of NAC on chronic bronchitis patients for a period of six months showed improvement in symptoms and decreased acute bronchial exacerbations (70-72).

Nevertheless, NAC has its disadvantages particularly in the area of topical administration. There is some difficulty in reaching more peripheral airways or poorly ventilated areas; there is also the risk of bronchospasm in asthmatic patients, and the need for aerosol-generating apparatus. Also, NAC can be too potent. It can overliquify the mucus in central airways yet underliquify the mucus in the periphery (54).

MESNA (2-Mercaptoethanesulfonic acid sodium salt)

Another cysteine derivative is the creamy white crystalline powder Mesna (73). Mesna has a dual action of reduction and ionization. The thiol group of mesna reduces the mucoprotein inter- and intrachain disulfide bridges, thereby reducing both the size of the mucin macromolecule and the number of constituents (74). The mesna molecule contains an ionized sulfonic group which increases the degree of hydration of the mucoprotein gel (75-77). Stiffness of the mucous structure is a result of the disulfide bridges. Mesna acts by breaking down the disulfide bridges and consequently reducing mucous viscosity (74). When administered in humans, mesna exerts profound mucolytic actions on respiratory tract secretions, thereby improving the viscosity of the sputum.

When compared to NAC, mesna demonstrates similar effects, but has a longer lasting action than NAC (73). Overall, mesna greatly reduces sputum

viscosity and improves airway clearability, thereby providing greater ease in expectoration.

DTT (DITHIOTHREITOL)

DTT is another cysteine derivative which has been considered as the most potent mucolytic thiol. Unfortunately it causes great irritation in the tissues and therefore cannot be used in clinical practice (76). DTT is rationally used in the cytology laboratory [as Sputolysin] to dissolve mucus away from cells thereby allowing only the cells to be examined.

SCMC (S-CARBOXYMETHYLCYSTEINE)

SCMC has been described as having mucoregulator properties as it demonstrates benefits on inflamed respiratory tract secretions (78). It has been suggested to increase the sulfur content of the mucus and reduce the bronchial secretions provoked by irritant aerosols (78). The sulfur on this molecule is attached to the carboxy group. Martin *et al.* showed SCMC to have no direct *in vitro* effect on mucus, presumably because of the blocked thiol (79).

UREA

Urea has long been recognized as a disassociating solvent which can break ionic and hydrogen bonds. Urea intervenes with hydrogen bonds which provide links between the oligosaccharide side chains of the neighbouring molecules, and thus causes disruption of hydrogen bonds. The disruption of the latter consequently reduces the physical entanglements between macromolecules, this process thereby reducing the viscosity of the sputum. Urea may also reduce the effects of DNA while separating physical entanglements.

In 1966, Waldron-Edward *et al.* (80) carried out an *in vitro* experiment using urea as a mucotropic agent. Concentrations of the urea examined varied from 1 to 8 M, it was reported that urea was effective only at 3 M concentration. Those concentrations less than 3 M had no significant mucolytic effect, while concentrations higher than 3 M would overliquify purulent sputum (81-83). For effective mucolysis, 0.2 g of urea for each 1 ml of sputum was used, though this is an immense amount of urea for nebulization. Therefore, because significant effects resulted only at such high concentrations, urea would be considered inappropriate for human usage. Waldron-Edward's study (80) was dismissed but many other investigators followed her findings in hopes of explaining the reasons for needing such high concentrations of urea. In 1974, Marriott and Richards (84) confirmed Waldron-Edward's findings explaining that when administered *in vitro*, the mucolytic action of urea increases as the concentration of urea increases from 5 to 8 mol/L. However, since toxic effects are generally concentration dependant, it is possible that one may avoid toxicity of urea by combining it with another mucotropic agent. The combination treatments of mucotropic agents are discussed in the section with the same heading to follow [pg. 21].

HYPERTONIC SALINE

"Normal Saline", i.e. 0.15 M [0.9%] sodium chloride, is approximately isotonic with serum; solutions with greater osmolarity are hypertonic, and solutions with lower osmolarity are hypotonic. *In vitro* hypertonic saline acts by changing the hydrogen and ionic bonding (Fig. II-1) while *in vivo*, it acts as an expectorant [promoting mucous secretion]. The interaction of a hypertonic solution with a mucosal surface results in both extraction of water from the tissue and the transfer of ions into the tissue until the osmotic pressure of the

solution becomes isotonic. The extra volume of water that is withdrawn from the tissue may result in either blockage of the small airways or dilution of the mucous gel, reducing its viscosity. For the purpose of this investigation, the effects of hypertonic saline as a mucotropic agent will be reviewed.

Hypertonic saline is believed to promote mucous clearance by extracting fluid from the respiratory tract epithelium, thereby increasing the volume and water contents of mucus, in effect causing bronchorrhea (85). The mechanism of action of hypertonic saline is as follows: upon administration of hypertonic saline by means of a nebulizer, water from the tissues will be shifted across the epithelial membrane to dilute the saline (86). This causes the airway fluid to approach normal tonicity; the diluted fluid is then eliminated from the respiratory tree by the mucociliary escalator or by coughing. However, if these clearance techniques fail, the saline will gradually be absorbed into the interstitium. One must take heed, that large quantities of hypertonic saline are not administered in the patient, as the result is a condition known as hypernatremia. Patients with renal insufficiency or impaired salt secretion are most likely to become hypernatremic.

As illustrated in Table II-1, hypertonic saline breaks up the ionic bond within the mucous gel. The efficacy of hypertonic saline is determined by the concentration administered. Concentrations of 20% saline could result in mucosal damage and hypernatremia; therefore, concentrations greater than 5% are rarely administered (87). However, hypertonic saline absorbs moisture as it moves through the airways; hence stimulating the growth of particles. By being deposited earlier and more proximally than hypotonic saline, hypertonic saline is instrumental in loosening secretions in the trachea and large bronchi. *In vitro* studies of hypertonic saline show that it is more effective in reducing mucoid sputum viscosity than is water (65).

Hypertonic saline separates the DNA molecules from the mucoprotein, making the DNA molecules susceptible to proteolytic enzyme digestion (88). Lieberman *et al.* (83) report that DNA cleavage by hypertonic saline increases as the ionic strength of the solution increases from 0.15 M. In 1978, Pavia *et al.* looked at the effect of hypertonic saline on chronic bronchitis patients (89). They found that, in comparison to normal saline, hypertonic saline [1.21 M] doubled the rate of mucociliary clearance with an increase in the weight of sputum expectorated. Wanner (90,91) explained this increase in mucociliary clearance by reporting that saline solutions stimulate mucociliary transport in both normal individuals and chronic bronchitis patients (92,93).

HYPOTONIC SALINE

Hypotonic saline [0.05 M] was used as a solvent for amiloride to provide a clinically effective drug concentration (94). Theoretically, aerosolizing hypotonic saline with or without amiloride could reduce the concentration of sodium and chloride in airway secretion, perhaps increasing the viscosity and elasticity even further. However, there is no evidence for this in clinical studies. In the amiloride study referred to above, the water content and rheological properties of the sputum before hypotonic saline aerosolization were not significantly different from those after aerosolization (95).

rhDNase (RECOMBINANT HUMAN DEOXYRIBONUCLEASE I)

Respiratory dysfunction in patients is due predominantly to the thick and viscous nature of the sputum (96). The elastic sputum is difficult to expectorate, and often contributes to reduced lung volumes and expiratory flow rates (97-99). Purulent secretions in patients suffering from respiratory dysfunction have similar characteristics to DNA, as an intrinsic property of DNA is to form thick

and viscous gels. High concentrations of DNA (up to 15 mg/ml) have been shown to be the leading cause of the tenacious and viscous nature of the sputum (44,100-102). By cleaving the DNA molecules, recombinant human deoxyribonuclease I (rhDNase) reduces sputum viscosity (101) transforming the tenacious sputum from a nonflowing gel to a flowing liquid within minutes. This change in the physical property of the sputum [nonflowing gel to a flowing liquid] helps the patients to remove sputum from their lungs, thereby improving their lung function. This reduction in viscosity of sputum is associated with a decrease in the size of the DNA molecules located within the sputum (101).

Recent multicenter studies of exacerbations in CF patients reported that short-term and long-term administration of rhDNase correlated with an improvement in pulmonary function (103,104). The use of rhDNase for the treatment of CF patients has been approved by Health and Welfare Canada and the Food and Drug Administration of the United States of America. The application of rhDNase in non-CF patients is under investigation (105,106).

GELSOLIN

F-actin is released from disintegrating inflammatory cells. It constitutes 10% of the total leukocyte protein (107) and develops long, protease-resistant, eminently viscoelastic filaments (108). Plasma gelsolin, which is a natural component of extracellular fluids, reduces the length of F-actin filaments (109). CF patients suffering from airway obstruction have present in their airways plasma proteins, within which there is usually gelsolin. However, there appears to be insufficient amounts of gelsolin present in the inflamed airways of the patient, thereby making it impossible to reduce the length of the F-actin filaments. Accumulation of the long F-actin filaments can cause mucus to be quite purulent and viscous similar to the excess concentration of DNA networks.

Vasconcellos *et al.* (109) reported a 58% decrease in the elastic shear modulus [the rate of reformation after distortion of the mucous gel] of CF sputum after treatment with gelsolin. Another study reported a significant decrease in the viscoelasticity index [$\log G^*$] and cough clearability index after treatment with gelsolin (110). Recently, in an *in vitro* study, I demonstrated that combined treatment with rhDNase and gelsolin was more effective in reducing the viscoelasticity of CF sputum than individual treatments with either agent, indicating mucolytic synergism between the two (111). A likely explanation for this action of gelsolin is that after disassociation, gelsolin obstructs the monomers from adding back onto filaments. These results substantiate reports of mucolytic action of gelsolin and confirm its efficacy in reducing the length of F-actin filaments. This shortening of F-actin filaments reduces not only the viscoelasticity and cough clearability of mucus, but also reduces the rate of formation of the distorted mucous gel. Further work is needed in this area as clinical studies with gelsolin have not yet been carried out.

OSCILLATION

The high frequency chest compression [HFCC] device (ThAIRapy®, American Biosystems, Inc.) is designed to mechanically assist or enhance pulmonary mucous clearance in CF. In HFCC, an inflatable vest is fitted on the patient, and connected to an airflow oscillation pump. A constant pressure of 50 cm H₂O is used to inflate the vest, and small oscillations at 10 to 25 Hz are superimposed on it. The resultant high frequency pressure oscillations compress the entire chest, causing air movements through the conducting airways, which are thought to break down mucus and assist its clearance from the lungs.

As illustrated in Table II-1, high frequency oscillations break up the physical entanglements by reducing the crosslinkages within the mucous gel. This disruption and disentanglement of the mucous macromolecules reduce the crosslinkages which bind the mucous glycoproteins, and thereby loosens the mucus to facilitate mucociliary clearance. However, oscillations may also break up the DNA molecules as these molecules have an inflexible helical structure, which make them more vulnerable to be broken by high frequency oscillation. Oscillation is most probably unable to break hydrogen and ionic bonds as both types of bonds are reversible and even if broken, they will reform.

Earlier studies have reported that the rate of mucociliary clearance is dependant on the degree of crosslinking (55); however a large reduction in crosslinkages will make the mucus too fluid, thereby obstructing mucociliary clearance. High frequency oscillation will transmit airflows through the lung tissue, vibrating the chest wall, and physically disrupting and disentangling the mucous macromolecules. High frequency oscillations could also enhance ciliary beating. The vagus is stimulated as a result of the vibration of the chest wall (112), via a reflex pathway, either through excitation of receptors in the chest wall or in the airway walls. Stimulation of the vagus would then result in acetylcholine release and in the increase of cilia beat frequency facilitating mucociliary transport (113).

Previous studies indicate that oscillating CF sputum (114,115) results in a decrease in spinnability. This decrease in spinnability may be explained by the reduction of polymer size induced through lysis of macromolecular backbone linkages (Fig. II-1). The decrease in spinnability of CF sputum correlated in previous studies (116) with increased transportability of mucus by ciliary and/or cough mechanisms. These changes in the physical properties of the sputum may therefore be considered potentially beneficial in the treatment

of CF patients.

The clinical value of HFCC is due to the high frequency airflow it generates through the lungs. This airflow not only reduces the viscosity of sputum but it also provides significant oscillatory airflow at the mouth through mucus-air-flow interaction, consequently provoking the patient to cough and expectorate a significant amount of sputum. Hence, the high frequency airflows are partially analogous to cough in provoking the movement of mucus mouthward which then leads to expectoration of sputum. King *et al.* (112) and Warwick *et al.* (117) both propose that HFCC is an excellent alternative to standard chest physiotherapy.

SURFACTANTS

Surfactants, such as Exosurf and bovine lipid extract solvent [bLES], are composed of dipalmitoylphosphatidylcholine [DPPC] and related phospholipids (118), and either surfactant associated protein or synthetic agents [which promote the spreading of surfactants]. Surfactants are found lining the alveoli; they keep the alveoli open during breathing. In newborns, the administration of exogenous surfactant improves the stability of the alveoli, thus permitting ventilation at lower pressures until maturation of natural surfactant production occurs. Currently, the usage of surfactants in the treatment of neonatal distress syndrome is routinely being applied (119,120).

In the airways, surfactant is produced by both the nonciliated bronchiolar cells (121) and bronchial mucous glands (122). By using *in situ* surface tension measurements, surfactants were identified in the airways of horses and sheep (123,124). Surfactants function by lubricating the airways for cough clearance. They also improve interaction with cilia thereby facilitating cilia beating. Hence, surfactants can improve both mucociliary and cough clearance, as well as

reduce surface tension and physical entanglements.

In vitro studies on surfactants have demonstrated that various surfactant preparations increase mucociliary transport (125-129). Recently, De Sanctis *et al.* (130) investigated the effect of Curosurf [a pulmonary surfactant] in improving mucociliary clearance *in vivo* in anesthetized dogs. They observed a 3 to 4 fold increase in tracheal mucous velocity [TMV] with Curosurf administration. The stimulation was attributed to the increased ciliary activity. Further studies are required to determine whether surfactants can improve mucociliary clearance in CF and chronic bronchitis patients.

Various studies have shown that the viscoelastic properties of airway mucus are altered by airway lipids (128,131). The change in the viscoelastic properties of airway mucus was mainly due to the surfactant-induced effect on the mucus rigidity. Sputum from CF patients demonstrates a correlation between changes in rheological properties and changes in phospholipid content (131); nevertheless, this does not mean that altering the phospholipid content will also alter the viscosity of CF sputum. De Sanctis *et al.* (130) reported that no change resulted in the viscoelasticity of dog tracheal mucus after administration of Curosurf, but a 4 to 6 fold increase was observed in mucous clearance.

The mucous bilayer [gel and sol] has been reported to be quite significant in mucociliary clearance by enhancing clearance through the sliding of the gel phase over the sol phase (123,124). It has been suggested that surfactants provoke the formation of bilayers between the mucus and the periciliary fluid (128). Not only do surfactants enhance the formation of the bilayer between the two airway phases, but they also augment the transfer of kinetic energy between the cilia and the mucous layer.

COMBINED TREATMENTS WITH MUCOTROPIC AGENTS

It has been illustrated above that treatments with a variety of individual mucotropic agents have been successful at improving the properties of mucus, thereby leading to increased mucous clearance. However, as illustrated in Table II-1, a combination of mucotropic agents can produce even greater effects upon mucolysis. For example, both rhDNase and Gelsolin are involved in breaking up the F-actin bonds. Vasconcellos *et al.* (109) found that both DNase and gelsolin had an additive effect in reducing the viscosity of CF sputum. Gelsolin is a protein capable of shortening the length of actin filaments, analogous to rhDNase which binds to monomeric actin and slowly depolymerizes the actin filaments (132). In an *in vitro* study, Vasconcellos *et al.* (109) combined these two mucotropic agents and reported that DNA may also reduce the viscosity of CF sputum through its action as an actin-binding protein (107). Actin inhibits the DNA-hydrolyzing activity of rhDNase (132); therefore the actin in CF sputum may have the same effect on DNA.

Both oscillation and the enzyme rhDNase can break apart the fragile DNA molecules found in CF sputum. Although the independent results on high frequency oscillation (133) and rhDNase (104) are very encouraging, to date these two therapies has not yet been researched to determine their additive effect on mucous clearance in CF patients. Therefore, in an *in vitro* study, I evaluated both the individual and combined effects of oscillation, saline, and rhDNase on CF sputum (114,115). The results from my *in vitro* study indicated that additive treatment with rhDNase and oscillation decreases spinnability of CF sputum significantly more than with either treatment alone [e.g. saline alone or oscillation alone] ($p < 0.01$).

As shown above, these *in vitro* results (109,115) demonstrate a maximal effect from the additive effects of mucotropic agents. This may suggest potential

superiority of combinations of chemical [e.g. rhDNase] and physical [e.g. oscillation] treatments on CF patients. Further studies are required to confirm these findings, to perform direct assessments of mucus clearability, and to extend the observations not only to a larger number of CF patients but also to patients with other types of pulmonary diseases.

SUMMARY

Airway mucus is the primary protective layer lining the conducting airways of the respiratory tract. It protects the underlying mucosa from dehydration while trapping inhaled particles and pathogens. Airway mucus originates mainly from two types of cells: goblet cells and glandular cells. Mucus is a viscoelastic secretion which has a unique molecular nature. It is composed of a number of different constituents: (1) water (90-95%), (2) mucous glycoproteins [also known as Mucins] (1-2%), and less than 1% is composed of (3) proteins, (4) lipids, and (5) low molecular weight ions.

The mucin macromolecules consist of a protein core with oligosaccharide units attached to it. The three-dimensional structure of the mucous gel is dependent upon a number of forms of bonding (Fig. II-1): (1) *disulfide bonds*, (2) *ionic bonds*, (3) *hydrogen bonds*, (4) *van der Waals forces*, and (5) *physical entanglements*. Additional crosslinkage is derived from extra macromolecules such as DNA, F-actin, and certain proteins.

The two-phase model of mucus (54,134-135) explains the mucociliary mechanism involved in removing mucus from the airways. Mucus flows on the sol layer and is transported by the cilia. The presence of this bilayer is important in mucociliary clearance because it facilitates the sliding of the gel phase over the sol phase.

Airway mucus has both benefits and disadvantages. The advantage of

mucus is that it serves as a protective layer trapping the bacteria, viruses, and foreign bodies in the conducting airways of the respiratory tract. The primary drawback is that the accumulation of airway mucus blocks the small airways causing airway obstruction. People suffering from diseases characterized by mucous hypersecretion [e.g. chronic bronchitis, CF] have excess mucus obstructing their airways.

Recent studies have suggested that maximal effects of mucolysis can be achieved through the combined treatments of both physical and biochemical agents (109,111,115). To date, the relationship between the individual and combined effects of mucolysis and clinical outcomes has not been determined. Therefore, the focus of my thesis will be to compare the individual and combined effects of mucolysis *in vitro* on mucous clearance in CF patients.

The effects of different approaches to mucolysis will be tested by studying their effects on different physical properties of airway mucus. This will be determined by measuring: 1) *viscosity and elasticity* (the basic parameters describing the liquid-like and solid-like properties of the mucous gel), 2) *spinnability and surface properties.*, 3) the effect on *clearance of airway mucus* by the two major relevant mechanisms: *ciliary action and airflow*.

The complex molecular nature of airway mucus can be simplified through the use of mucotropic agents. Mucotropic agents are involved in disrupting the crosslinkages and bonding within the mucous gels, a process known as mucolysis. The individual and combined effects of seven mucotropic agents: (1) Nacystelyn (the lysine salt of N-acetylcysteine), (2) Urea, (3) Hypertonic Saline, (4) rhDNase, (5) Gelsolin, (6) Oscillation, and (7) Surfactants [such as Exosurf, a synthetic mixture, or bLES, a bovine lipid extract preparation] were examined. Each of these mucotropic agents disrupt a separate bond within the mucous gel (Table II-1). The overall function of the aforementioned mucotropic agents is to

reduce the number of mucin macromolecules, thereby reducing entanglements. If successful results are obtained, then these results would support trials involving combined treatment with mucolytic agents on CF patients, aimed at decreasing patient morbidity and increasing cost-effectiveness of treatment.

In conclusion, a variety of mucotropic agents is involved in reducing the viscoelasticity of the mucous gel. Previous *in vitro* and *in vivo* studies have been successful utilizing individual mucotropic agents (e.g. 104,114), and a few studies, including ours (109,115), indicate that combined mucolytic treatments have greater potential. Nevertheless, additional studies are required to confirm these findings, and to further define the additive effects of mucolysis with combined treatments of physical and biochemical mucotropic agents.

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Types of Bonds Occurring in a MUCOUS GEL

1. COVALENT BONDS

- primarily S-S bonds (intra- and inter-molecular)

2. IONIC BONDS

- mucin macromolecules have both positive and negative fixed charges which are capable of interacting

3. HYDROGEN BONDS

- are due to the oligo-saccharide side-chains

4. VAN DER WAALS' FORCES

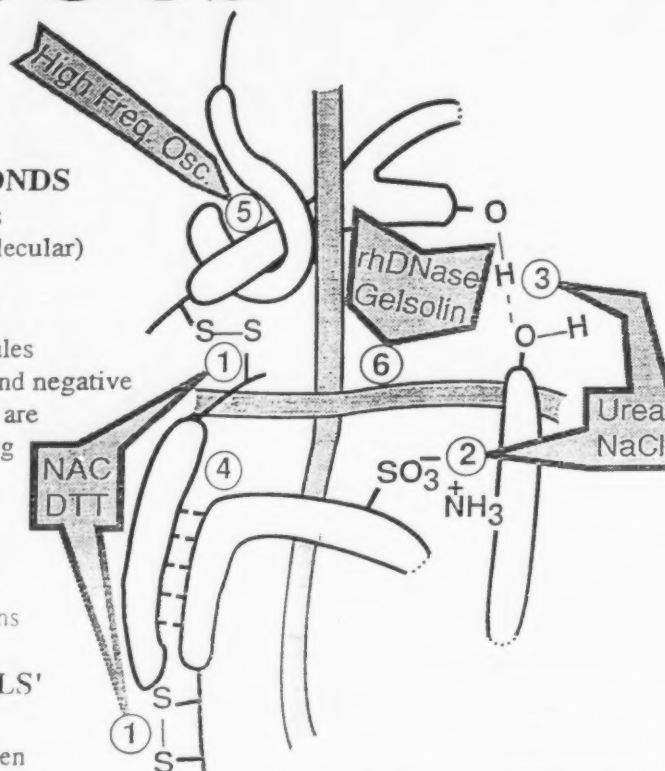
- interdigitation between oligosaccharide molecules may be important

5. INTERMINGLING

- physical entanglements

6. EXTRACELLULAR DNA & F-ACTIN

- parallel network formation in infection



Each of these types of bonds is subject to Mucolysis

Figure II-1

Diagram illustrating the types of bonding in a mucous gel, and site of action of various mucolytic treatments.

Components of the Mucous Gel

Sources of Bonding Mucotropic Agents	DNA	F-actin	Disulfide Bonds	Ionic Bonds	Hydrogen Bonding	Surface Tension	Physical Entangle
rhDNase	X	?					X
Gelsolin	?	X					X
N-acetyl-cysteine			X				X
Hypertonic Saline				X			X
Urea	X			X	X		?
Exosurf/ bLES						X	X
Oscillation	?	?	?				X

X = expected interaction ? = possible interaction

Table II-1

Table indicating the expected and possible sites of action of various mucolytic treatments on the molecular components of the mucous gel.

Chapter III

**EFFECTS OF COMBINED TREATMENT WITH rhDNase AND
AIRFLOW OSCILLATIONS ON SPINNABILITY OF CYSTIC FIBROSIS
SPUTUM *IN VITRO***

INTRODUCTION

Purulent secretions in cystic fibrosis (CF) lung disease tend to be more viscoelastic and less clearable by ciliary action (1,2). Comprised lung clearance in CF correlates with lung function (3), and bacterial infection in the CF airways worsens these conditions by promoting increased inflammatory responses with mucus hypersecretion and neutrophil migration into the airways (4). Elevated concentrations of high molecular weight neutrophilic DNA have been found in CF sputum (5-8) and are believed to be the major cause of the tenacious and viscous nature of purulent CF sputum. Recently approved treatment with recombinant human deoxyribonuclease I (rhDNase - Pulmozyme®) improves lung function and decreases the frequency of exacerbations in some CF patients (9,10). On the other hand, standard chest physiotherapy (CPT) has long been included in the management of CF lung disease; however, a mechanical alternative to CPT – high frequency chest compression (HFCC - ThAIRapy®) – was reported to be more effective in terms of patient compliance and long-term lung function outcomes (11), or short-term sputum production (12).

Both DNase and HFCC exert their action through different mechanisms (enzymatic cleavage *versus* physical disruption), which constitutes a rationale for the possibility that they have an additive effect on the physical properties of airway secretions. DNase decreases sputum viscosity and/or elasticity both *in vitro* and *in vivo* by cleaving high molecular weight DNA present in CF airway secretions (7,13-16). A clinical study with HFCC resulted in significantly greater sputum production with increased water content after 1 hr collection in comparison to standard CPT (12), while high frequency airflow oscillations resulted in both reduction of spinnability and viscoelasticity of mucous gel simulants (17).

Although both rhDNase and HFCC are currently being used in patients with CF lung disease, to date no work has been done on examining the possible additive effect of combining these two therapies in the treatment of CF. Therefore, the objective of this *in vitro* pilot study was to determine whether combined treatment with DNase and airflow oscillations would have a greater effect on the physical properties of CF sputum than either treatment by itself. To answer this question the measurement of spinnability as a simple, bedside-like test was used; this measurement has been reported to significantly decrease and associate with improvement of lung clearance after amiloride treatment in cystic fibrosis (18).

MATERIALS AND METHODS

Subjects - Sputum samples were collected from eight patients with CF by voluntary expectoration during a routine clinical visit. The patients (6 men and 2 women, age 17 to 39, mean 24 years) were all infected with *Pseudomonas aeruginosa*, and were treated with steroids, antibiotics, and bronchodilators as required. None of the patients had been treated with DNase up to the time of sputum collection, and none had used HFCC for physiotherapy, or were using any mucolytic preparation. Approval to collect and use sputum for *in vitro* analysis was obtained from the University of Alberta Research Ethics Board.

Study Design - Aliquots of each sputum sample (0.4 g) were subjected to the following treatment protocols: *i*) Treatment with *ca.* 5 µg/ml rhDNase (Pulmozyme®, Genentech, Inc.). To achieve this final concentration, 10% of the volume of the aliquot containing 10 times the target concentration of drug in normal saline (0.9% NaCl) was layered over the sputum sample without stirring, and incubated at 37°C for 15 minutes; *ii*) An aliquot was inserted into an open-ended, 4-mm (inner diameter) vinyl tube connected to a device producing oscillating airflow at 27 Hz (high frequency airflow oscillation) (17). The airflow magnitude was similar to that produced by a commercial HFCC device (ThAIRapy®, American Biosystems, Inc.) (11), when corrected for the difference in cross-sectional area between the tube and the human trachea. The air in the tube was oscillated over the sample for two 15-minute intervals; *iii*) As a negative control, an aliquot was inserted into an identical open-ended tube for 30 minutes without applying oscillations; *iv*) As a positive control to test the effect of dilution, a separate aliquot was treated with 10% of the volume of normal saline, and incubated at 37°C for two 15-minute periods.

To evaluate the combined effects of oscillation and DNase, protocols i and ii were combined. DNase was administered to an aliquot of the sputum sample that was then oscillated for two 15-minute periods. Similarly, protocols ii and iv were combined to observe the combined effects of saline and oscillation. Ten percent volume of saline was administered to a separate aliquot of the CF sputum sample, following which the sputum sample was oscillated for two 15-minute periods.

For each treatment protocol, spinnability measurements were done prior to any treatment (baseline), and then after 15 and 30 minutes of application of the treatment. Three spinnability readings per aliquot were taken, and the arithmetic mean of the three readings was calculated.

Spinnability Measurements on CF Sputum - Spinnability is the thread forming ability of mucus under the influence of low speed elastic deformation. Using a Filancemeter (type 04, SEFAM) (19), a 20 to 40 μ L mucus sample is stretched at a distraction velocity of 10 mm/sec. An electric signal conducted through the mucus sample is interrupted at the point where the mucus thread is broken; the length of this thread is known as the mucus spinnability (measured in mm). The longer the thread the greater the viscoelasticity of the sputum sample; the shorter the thread the less the viscoelasticity of the sputum sample.

Statistical Analysis - Data from each protocol are presented as mean $\pm$ standard deviation (SD). To analyze the significance of changes in spinnability after administration of rhDNase and oscillation, the sputum from each patient served as its own control. The two-tailed paired t-test was also used to determine the overall significance of the changes from baseline for spinnability after different types of treatment.

RESULTS

In vitro treatment of CF sputum with DNase or airflow oscillation resulted, after 30 minutes, in a mean decrease in spinnability from baseline of 59% and 34%, respectively, whereas the mean decrease in spinnability after combined treatment of DNase and oscillation was 77%. Compared to their respective controls, however, the effects of DNase and oscillation were quite similar, i.e. DNase vs. saline at 30 minutes resulted in a 42% decrease in spinnability, while oscillation vs. negative control gave a 41% decrease.

Individual Effects of Mucolysis [Figure III-1] - Administration of DNase for 15 minutes (line 1d) decreased spinnability significantly more than either oscillations or saline ($p = 0.04$ and $p = 0.02$ respectively); the difference between DNase and saline was also significant at 30 minutes (5.21 ± 0.48 vs. 8.99 ± 1.51 mm, $p < 0.01$). Oscillation over 15 minutes (line 1c) decreased spinnability similarly to incubation with normal saline (line 1b) over the same period of time, both significantly lower than baseline (10.2 ± 1.31 mm for oscillation and 9.34 ± 1.79 mm for saline vs. 12.6 ± 2.99 mm for baseline). There was no significant difference between 15 and 30 minutes for saline, whereas spinnability decreased significantly further for the additional 15-minute period of oscillations ($p < 0.01$). Spinnability of the control sample with no treatment (line 1a) increased nonsignificantly (to 14.1 ± 1.1 mm at 30 minutes).

Combined Effects of Mucolysis [Figure III-2] - The combination of DNase and oscillation over a period of 15 and 30 minutes (line 2d) decreased spinnability significantly more than DNase alone (line 2c) over the same period of time (2.74 ± 0.20 vs. 6.54 ± 0.33 mm at 15 minutes, and 2.94 ± 0.21 vs. 5.21 ± 0.22 mm at 30 minutes, each $p < 0.01$). The combined administration of saline and

oscillation (line 2b) decreased spinnability more than oscillation by itself (line 2a), and almost as much as the administration of DNase alone (6.70 ± 0.64 vs. 5.21 ± 0.22 mm at 30 minutes).

DISCUSSION

This *in vitro* study demonstrates that treatment of CF sputum with either rhDNase or high frequency oscillation decreases the spinnability when compared to their respective control. The combination of DNase and oscillation decreases the spinnability more than either treatment alone.

The changes in spinnability of oscillated CF sputum are consistent with the results reported in our previous study involving mucous simulant gels (17), where we also observed a decrease in spinnability as well as in viscoelasticity. Moreover, an earlier study in dogs also demonstrated a decrease in mucus viscosity with HFCC (20). Since a minimum chain length and degree of inter- and intramolecular crosslinking are necessary for spinnability (19), it has been suggested that the decrease in spinnability of the gel polymers after oscillations is due to physical rupture of macromolecular backbone linkages and mechanical disruption of entanglements (17).

The further decrease in spinnability of CF sputum treated with DNase could be related to its mechanism of action. DNase breaks down high molecular weight DNA (which makes the sputum viscous and tenacious) to low molecular weight DNA, thereby reducing the viscosity of purulent CF sputum (7). This has been confirmed in several *in vitro* studies (13-15), as well as *in vivo* by Shah and colleagues (16). By analogy to the mechanical disruption of mucous gels by oscillation and the effect on spinnability, the decrease in molecular length of DNA by DNase could also account for the decrease in spinnability observed here.

In the present study, the results demonstrate a maximal reduction in spinnability from combining DNase and oscillation. A previous clinical study by App and coworkers (18) found that an acute treatment with amiloride aerosol reduced CF sputum spinnability; this change was associated with an increase

in mucociliary and cough clearance with the amiloride treatment. Furthermore, the decrease in spinnability correlates with increased clearability by cough mechanism as confirmed by *in vitro* studies (21). Thus, the changes observed in the present investigation may be considered potentially beneficial in the treatment of CF lung disease.

In a study of chronic bronchitis (CB) sputum, spinnability was reported to correlate positively with transport rate on the frog palate (19). However, both purulent and mucoid samples that covered a wide range of spinnabilities (from approximately 15 to 100 mm) were used in this analysis, and the lower spinnability values were associated with purulence and high viscoelasticity. The samples used in this study were considered to be more viscoelastic, as is expected of purulent secretions from infected CF airways (22), and pretreatment spinnability values were in the range of the lowest values reported by Puchelle and colleagues (19). Furthermore, this study evaluated an acute treatment of CF sputum *in vitro*, where the degree of purulence and the chemical properties of the mucus could not change, while the former study was a cross-sectional study of CB sputum with a variety of degrees of purulence. Thus, it is unlikely that the described positive correlation is applicable to this study.

Despite some limitations of this study, such as the absence of other rheological measurements or determination of mucociliary clearance, spinnability seems to be a useful screening method for assessing acute mucokinetic treatments. However, further studies to delineate the effects of combined treatments on other physical and transport characteristics of mucus, such as viscoelasticity, adhesivity, and inherent ciliary and cough clearability, are indicated. In conclusion, this *in vitro* study demonstrates that the combined effects of DNase and oscillation decreases the spinnability of CF sputum significantly more than individual treatments, thereby suggesting potential

superiority of combinations of chemical and physical treatments on CF patients. Since both forms of physical and chemical therapy, i.e. HFCC and rhDNase, are used in CF patients, the effects of combined therapies should be investigated concurrently in clinical studies.

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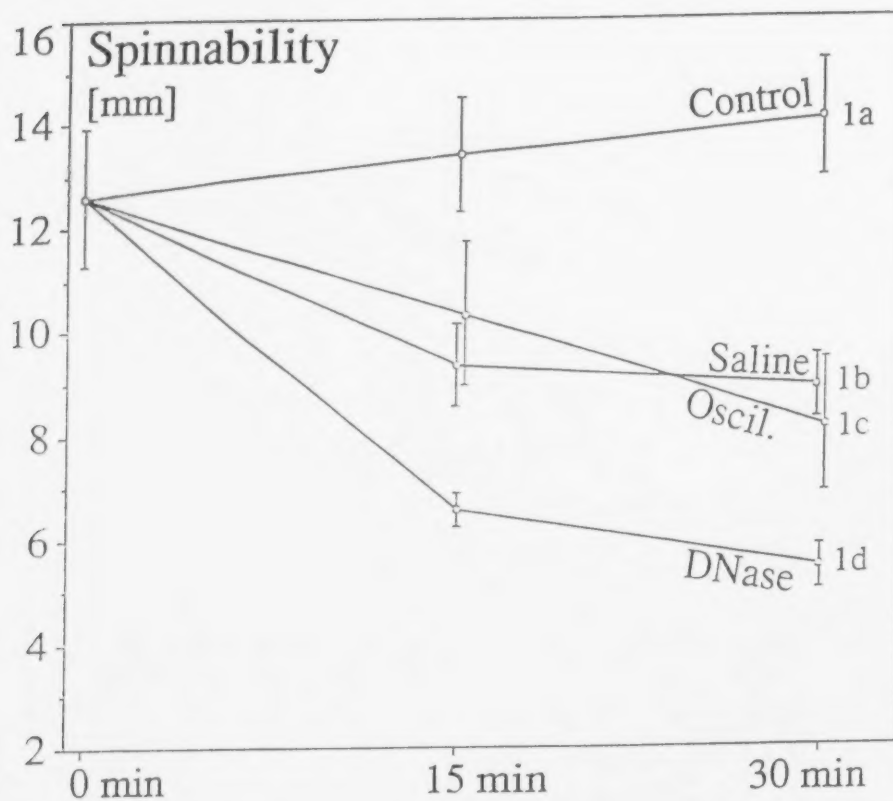


Figure III-1

Individual effects of saline, oscillation, and rhDNase on spinnability of CF sputum. Spinnability values (mean $\pm$ SD) for different treatment protocols and controls are shown in this figure. Different treatment protocols are identified by letters and numbers which correspond to the descriptions in the Results section.

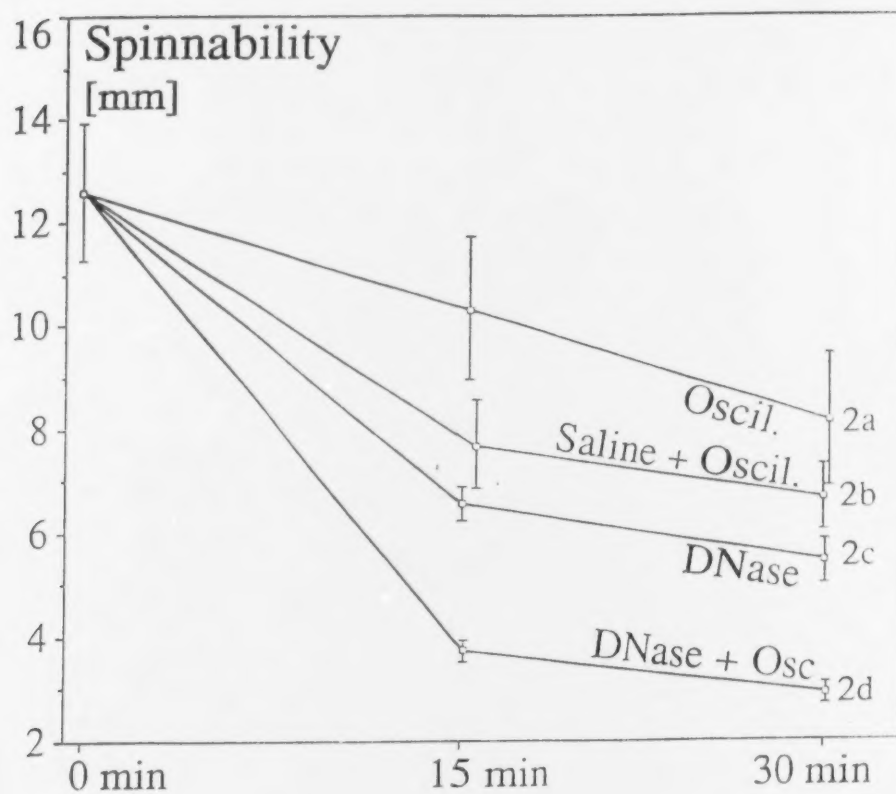


Figure III-2

Combined effects of saline, oscillation, and rhDNase on spinnability of CF sputum. Different treatment protocols are identified in a similar manner as described in Figure III-1, and correspond to the descriptions in the Results section.

Chapter IV

**MUCOLYTIC SYNERGISM IN CYSTIC FIBROSIS (CF) SPUTUM
WITH COMBINED rhDNase AND GELSOLIN TREATMENT *IN VITRO***

INTRODUCTION

Cystic fibrosis (CF) is characterized by the accumulation of thick and purulent airway secretions (1) which cause impaired lung clearance. These secretions are difficult to clear by mucociliary and cough mechanisms, thereby plugging the airways and resulting in reduced lung volumes and expiratory flow rates (2-4). A reduction in the viscosity and elasticity of viscous sputum could transform it from a nonflowing gel to a flowing liquid; this physical change in the sputum could then enhance expectoration, thus improving airway function of the CF patient.

Previous studies have shown that the presence of high concentrations of DNA (up to 15 mg/ml) in airway secretions is a major cause of the viscosity of CF sputum (5-8). The licensed mucolytic, recombinant human deoxyribonuclease I (rhDNase - Pulmozyme®) has been reported to cleave high molecular weight DNA molecules, consequently reducing sputum viscosity (5) and spinnability (9).

Vasconcellos *et al.* (10) suggested that polymers of F-actin derived from leukocyte cytosolic structural filaments may contribute to both the rigidity of CF sputum and to neutrophilic DNA. Janmey *et al.* (11) reported that the viscosity of F-actin solutions was proportional to the actin filament length. Gelsolin, a capping protein that severs F-actin filaments (10), has been shown to reduce the viscosity of CF sputum.

Independent *in vitro* studies on rhDNase (9,12-14) and a clinical study on rhDNase (15) confirm its usefulness as a mucolytic treatment for CF. Also, Vasconcellos *et al.* (10) have reported that gelsolin treatment, *in vitro*, improves the rheology of CF sputum. Yet, to date, no work has been done to observe the synergistic effects of rhDNase and gelsolin on the spinnability and viscoelasticity of CF sputum. Thus, the objective of this study was to compare *in*

vitro the synergistic effects of rhDNase and gelsolin on the rheological properties of CF sputum.

MATERIALS AND METHODS

Subjects - Sputum samples were collected from eleven patients with CF by voluntary expectoration during a routine clinical visit. The patients (14 to 27 years, mean age 22 yrs) were all infected with *Pseudomonas aeruginosa* and treated with steroids, antibiotics, and bronchodilators as required. The patients had been exposed neither to rhDNase nor to gelsolin up to the time of sputum collection, and none were using any other type of mucolytic preparation. Approval to collect and use sputum for this *in vitro* analysis was obtained from the University of Alberta Research Ethics Board.

Study Design - Aliquots of each sputum sample (0.2 to 0.4 g) were subjected to four different treatment protocols: *i*) incubation with rhDNase [Pulmozyme®, Genentech, Inc.] in normal saline to achieve *ca.* 2.5 µg/mL final concentration; *ii*) incubation with gelsolin [Biogen, Inc.] in normal saline to achieve 8.4 µg/mL final concentration; *iii*) negative control with no treatment (e.g. rhDNase, gelsolin); *iv*) positive control, to test the dilution effect, incubated with 10% normal saline. The final total concentration for each active treatment was *ca.* 100 nM. The samples in protocols *i*, *ii*, and *iv* were incubated at 37 °C for 30 minutes.

To observe the combined effects of gelsolin and DNase, protocols *i* and *ii* were combined at half the concentration of each agent (50 nM) then incubated at 37°C for 30 minutes. The concentrations of mucotropic agents (100 nM total concentration) were chosen on the basis of preliminary experiments to achieve a less than maximal reduction in spinnability in order to observe additive or synergistic effects. The incubation conditions (30 minutes at 37 °C) were observed in the previous chapter to produce an approximate plateau of effect on spinnability.

For each treatment protocol, spinnability, by filancemeter, and viscoelasticity ($\log G^*$), by magnetic microrheometer, were measured and mucociliary clearability index (MCI) and cough clearability index (CCI) were calculated prior to any treatment (baseline), and then after 30 minutes of application of the treatment. Three spinnability readings per aliquot were taken, and the arithmetic mean of the three readings was calculated.

Spinnability Measurements on CF Sputum - Spinnability is the thread forming ability of mucus under the influence of low speed elastic deformation. The spinnability of CF sputum samples was measured using a Filancemeter (type 04, SEFAM) (16) modified to accommodate small sample volumes (17), in which a 20 to 40 μL mucus sample is stretched at a distraction velocity of 10 mm/sec. An electric signal conducted through the mucus sample is interrupted at the point where the mucus thread is broken; the length of this thread is known as the mucus spinnability (measured in mm). The longer the thread the greater the viscoelasticity of the sputum sample; the shorter the thread the less the viscoelasticity of the sputum sample.

Rheological Measurements on CF Sputum - Mucus can be considered as a viscoelastic fluid, since it exhibits both viscous and elastic properties. Viscosity and elasticity of microliter quantities of mucus (18,19) are measured by means of a magnetic microrheometer.

A 100 μm steel sphere is placed within a 2-5 μL sample of mucus. An electromagnet moves this sphere and projects it onto a pair of photocells via a microscope. The motion of the sphere is plotted against the driving force of the magnet on an oscilloscope, forming an ellipse. From this ellipse, the parameters of mucus viscoelasticity can be determined: i) G^* , or mechanical impedance, is

the vector sum of viscosity and elasticity and reported in log scale; it can also be termed the rigidity factor; ii) $\tan \delta$, or loss tangent, is the ratio of viscosity to elasticity.

Specifically, two derivative parameters were calculated from *in vitro* relationships: MCI and CCI. The MCI, indicating clearability by normalized ciliary function, was computed from G^* and $\tan \delta$ at 1 rad/s, and the CCI was computed from G^* and $\tan \delta$ at 100 rad/s. Both indices relate negatively with log G^* . MCI relates negatively with $\tan \delta$, but CCI relates positively with it. The respective formulae for MCI and CCI (20) are the following:

$$(1) \text{ MCI} = 1.62 - (0.22 \times \log G^*1) - (0.77 \times \tan \delta 1)$$

$$(2) \text{ CCI} = 3.44 - (1.07 \times \log G^*100) + (0.89 \times \tan \delta 100)$$

Based on model studies, both the MCI and the CCI can be used to predict the effect of mucus clearance, both by ciliary action and cough mechanism (21).

Electrolyte Content - The analysis of the electrolyte content was carried out as follows: An aliquot of the sputum sample (minimum 30 g) was suspended in 300 μl of urea, vortexed for 5 minutes, and incubated overnight at 4°C. Following incubation, the sample of sputum was centrifuged for 20 minutes at 10,000 RPM. The supernatant was subsequently analyzed with ion-selective electrodes for sodium and potassium content (Sodium/Potassium Analyzer Nova 1; Nova Biomedical), and a chloride meter (Model 920M; Cornfield) was used to measure the chloride content (22). Only the control samples were analyzed.

Dry and Wet Weight - The solids content of CF sputum samples (control samples only) were calculated in the following manner: The initial weight of the samples were taken (Mettler analytical balance) following which the samples were microwaved for 30 minutes at 750 watts. The weight of the dessicated sample was taken and the latter was divided by the initial weight of the sample and multiplied by 100 to obtain the ratio of dry and wet weights as the percent solids content (23).

Statistical Analysis - Data from each protocol are presented as mean $\pm$ standard deviation (SD). To analyze the significance of changes in spinnability and the other viscoelastic parameters after administration of rhDNase and gelsolin, the sputum from each patient served as its own control. For each treatment, we determined the overall significance of the changes from baseline with a two-tailed paired t-test. The two-tailed paired t-test was also used to determine the differences between spinnability, MCI, and CCI after different types of treatment.

RESULTS

Spinnability Measurements

In CF sputum, individual treatment with DNase caused a greater mean decrease in spinnability [from baseline] (63%) than that with a singular treatment with gelsolin (56%). The mean decrease in spinnability after combined treatment with DNase and gelsolin was 73%. When compared to their respective controls (i.e. incubation with saline), a similar pattern was observed in mean reduction in spinnabilities. At thirty minutes, gelsolin vs. saline gave a 49% decrease, DNase vs. saline gave a 57% decrease, and combination vs. saline resulted a 69% decrease in spinnability. These results on spinnability are presented in Table IV-1.

Individual Effects of Mucotropic Agents - As illustrated in Figure IV-1, the administration of gelsolin (100 nM final concentration) over 30 minutes decreased the spinnability more than saline treatment (6.19 ± 3.47 for gelsolin vs. 12.26 ± 2.18 mm for saline, respectively, $p = 0.0003$). DNase (100 nM final concentration) also decreased spinnability significantly more than saline treatment for 30 minutes (5.21 ± 2.82 for DNase vs. 12.26 ± 2.81 mm for saline, respectively, $p < 0.0001$). Despite the fact that the decrease in spinnability with equimolar DNase was greater than that with equimolar gelsolin (5.21 ± 2.82 for DNase vs. 6.19 ± 3.47 mm for gelsolin, respectively), the difference did not achieve statistical significance ($p = 0.16$). Saline has a small but significant effect on spinnability (12.26 ± 2.18 for saline vs. 14.12 ± 1.89 mm for baseline, respectively, $p = 0.007$)

Combined Effects of Mucotropic Agents - The combined administration of DNase and gelsolin (50 nM of each agent) showed a significant decrease in

spinnability when compared to incubation with normal saline over 30 minutes (3.80 ± 1.64 for combination vs. 12.26 ± 2.81 mm for saline, respectively, $p < 0.0001$) (Fig. IV-1). The combined administration of DNase and gelsolin, over a period of 30 minutes, decreased spinnability significantly more than both the administration of DNase alone (3.80 ± 1.64 for combination vs. 5.21 ± 2.82 mm for DNase, respectively, $p = 0.03$) and the administration of gelsolin alone (3.80 ± 1.64 for combination vs. 6.19 ± 3.47 mm for gelsolin, respectively, $p = 0.005$).

Rheology and Clearance Indices

As indicated in Table IV-1, singular treatment with gelsolin did not demonstrate any significant changes in the index of sputum rigidity, $\log G^*$ at 1 rad/s, in comparison to saline (1.75 ± 0.48 for gelsolin and 1.72 ± 0.56 for saline, $p = 0.91$). Both the singular administration of DNase and the combined administration of gelsolin and DNase reduced the $\log G^*$ at 1 rad/s nonsignificantly when compared to saline (1.64 ± 0.36 for DNase vs. 1.60 ± 0.45 for DNase + gelsolin, $p = 0.52$ for DNase and $p = 0.61$ for DNase + gelsolin, respectively). Also, as illustrated in Figure IV-2, individual treatments with DNase and gelsolin, and the combination of gelsolin and DNase produced nonsignificant changes in MCI in comparison with saline ($p = 0.51$ for DNase, $p = 0.50$ for gelsolin, and $p = 0.86$ for DNase + gelsolin, respectively). Parallel results were also demonstrated with the CCI (Fig. IV-3) in comparison to saline ($p = 0.99$ for DNase, $p = 0.77$ for gelsolin, and $p = 0.45$ for DNase + gelsolin, respectively).

Dog mucus treated *in vitro* with rhDNase showed a small reduction in spinnability (13.05 ± 0.50 mm for control vs. 9.60 ± 0.28 mm for DNase). DNase decreased $\log G^*$ at 1 rad/s and increased both the MCI and the CCI non-

significantly ($p = 0.13$ for $\log G^*$, $p = 0.41$ for MCI, and $p = 0.37$ for CCI, respectively).

Electrolyte and Solids Content

The baseline sodium content of CF sputum samples was 110.96 ± 19.65 mmol/L while the baseline solids content was $8.88 \pm 2.78\%$. These results are in line with previously published data on CF sputum solids and electrolyte content (22). The sputum potassium and chloride content appeared to be somewhat lower (13.09 ± 1.76 mmol/L for potassium, 33.72 ± 11.89 mmol/L for chloride) than other reported data.

The data were examined for possible correlations between baseline electrolyte and solids content and the response to the mucotropic agents (gelsoin, DNase, and the combination of the two agents). There were no significant correlations found between changes in $\log G^*$, and baseline solids and sodium content.

DISCUSSION

Singular treatments with rhDNase or gelsolin on CF sputum demonstrated a significant decrease in spinnability when compared to CF sputum treated with baseline. However, in comparison to singular treatments, the combined treatment of gelsolin and rhDNase, at half the concentration of each mucotropic agent, showed an even larger, statistically significant reduction in spinnability of CF sputum (Table IV-1). This is an indicator of synergy in action between the two mucotropic agents.

The decrease in spinnability of gelsolin treated CF sputum is related to the mechanism of action of gelsolin. Ten percent of the leukocyte protein is composed of actin (24); these actin molecules form long protease-resistant, highly viscoelastic filaments (11, 25-26). The protein gelsolin is an actin binding protein which rapidly severs the noncovalent bonds between monomers within a filament (27). By breaking these viscoelastic filaments, gelsolin is able to reduce the viscosity of CF sputum. This correlates with what we found in the present study.

Large concentrations of DNA, released from degenerating polymorphonuclear leukocytes, bacteria, and cell debris, are found in the sputum of CF patients (7, 28-29). DNA has been reported to be directly associated with increasing sputum viscosity and binding to protein to prevent proteolysis (29). Addition of rhDNase depolymerizes DNA molecules, reducing the adhesiveness of sputum and enhancing sputum clearability (30). The results of this present study confirm the effectiveness of rhDNase in improving sputum rheology.

The combined treatment of rhDNase and gelsolin at similar total mucolytic concentrations (50 nM of each mucolytic agent) decreased spinnability significantly more than the singular treatments of rhDNase or

gelsolin alone. The spinnability results of the combination treatment was more than an additive effect – it was a synergistic effect of rhDNase and gelsolin. As rhDNase has been reported to bind and slowly depolymerize actin filaments (31,32), the possibility of rhDNase, as an actin binding protein, in combination with gelsolin to facilitate the clearance of filamentous actin was considered. Gelsolin, which binds to monomeric actin, is a protein capable of shortening the length of actin filaments (32), analogous to the action by rhDNase on DNA. Actin also inhibits the DNA-hydrolyzing activity of rhDNase (32); therefore, by binding to actin, the action of rhDNase in CF sputum may be enhanced. Hence, in this *in vitro* study, the combined treatment with rhDNase and gelsolin greatly reduced the viscoelasticity of purulent CF sputum, indicating synergy between the two mucotropic agents.

In the present study, singular and combined treatments of rhDNase and gelsolin significantly reduced mucus spinnability, yet were nonsignificant for both MCI and CCI (Figures IV-2 and IV-3). Perhaps this may be explained by the diffusion capacity of the mucotropic agents to enter the mucous gel. Rheological properties are determined not only by the structure and concentration of different forms of macromolecules, but also by the interaction of these molecules.

The difference in results of spinnability, MCI, and CCI after individual and additive treatments of gelsolin and rhDNase may also be explained by the sensitivity of the different techniques which were used to measure the rheological parameters. Spinnability was measured using a Filancemeter (type 04, SEFAM) (12), and MCI and CCI using a magnetic microrheometer. Spinnability, which is related to the normal stress difference, has a much higher power dependence on detecting changes in molecular weights of sputum samples than does the magnetic microrheometer (6.8 power vs. 3.4 power)

(33). Both geisolin and rhDNase synergistically act to cleave and thereby reduce the weight of DNA molecules. Spinnability is useful in measuring this change in the DNA content of sputum samples. However, the magnetic microrheometer is not as sensitive in measuring changes in molecular weight. Therefore, in our study, spinnability was the better of the two techniques for measuring rheological changes that were due to mucolytic treatments (i.e. gelsolin and rhDNase) which reduced the high molecular weight DNA components of sputum.

Dog mucus treated *in vitro* with rhDNase showed a small reduction in spinnability and a non-significant decrease in $\log G^*$. A probable explanation for these results is due to the variation in molecular composition of mucus from different study populations. Sputum obtained from CF patients with prolonged infection will contain DNA, F-actin, and proteases, making CF sputum highly purulent and viscous. On the other hand, tracheal mucus obtained from healthy dogs will be more homogeneous and free of by-products of infection, making dog mucus less viscous than CF sputum and less responsive to DNase. Therefore, treatment with rhDNase did not demonstrate any significant changes in the viscoelasticity of dog mucus.

No distinct relationship was demonstrated between baseline data on percentage solids and electrolyte content of sputum in relation to either baseline rheology or response to mucolytic treatment. A possible explanation for this may be that the rheology of these types of sputum samples are much more dependent on the contributions of added network polymers such as F-actin and DNA. Since both DNA and F-actin are not directly related to factors such as water content and electrolyte content (both of which affect the normal glycoprotein gels), no change was demonstrated in the rheology.

When comparing the effects of two mucotropic agents, the relative potency of each agent is evaluated. The nature of the sputum sample will influence the potency of a mucotropic agent. Depending on the nature of infection, some sputum samples could have higher DNA or actin content. This helps to explain the potency of gelsolin and rhDNase in various different studies. *In vitro* studies of additive treatment with gelsolin and rhDNase report variations in evaluating the potency of the agents: Vasconcellos *et al.* (10) found gelsolin to be a more potent agent, and Tomkiewicz *et al.* (34) reported DNase to be a more potent agent, whereas, in the present study, the relative potency of the two agents were found to be similar. This difference in evaluating potency of rhDNase and gelsolin may be due to the distinct nature of the sputum samples each investigator studied, and the degree of infection within the respective samples. Regardless of the potency of the agents, we have demonstrated that a synergistic effect results upon combining gelsolin and rhDNase.

Therefore, this *in vitro* study demonstrates that combined treatment with rhDNase and gelsolin: (i) decreases the spinnability significantly more than individual treatment with either rhDNase or gelsolin alone; (ii) nonsignificantly decreases the $\log G^*$ (the principal viscoelasticity index); and (iii) did not demonstrate any significant changes in either the CCI and MCI. These results on the improvement in sputum rheology correlated in previous studies (10) with a decrease in viscoelasticity of CF sputum. Therefore, these results may be considered potentially beneficial, and suggest that the additive treatment of rhDNase and gelsolin deserves consideration for therapy in CF patients.

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Rheological effects of rhDNase and Gelsolin treatment on CF sputum

	BASILINE	SALINE	rhDNase	GELSOLIN	COMBINATION
Spinnability (mm)	14.1 ± 1.89	12.3 ± 2.81	5.2 ± 2.82 *§	6.2 ± 3.47 *§	3.8 ± 1.64 *
Viscoelasticity (log G* @ 1 rad/sec)	1.78 ± 0.45	1.72 ± 0.56	1.64 ± 0.36	1.75 ± 0.48	1.60 ± 0.45

[*p < 0.001 in comparison with saline by paired t-test; §p < 0.05 in comparison with combination]

Table IV-1

Singular and combined effects of rhDNase and Gelsolin on spinnability (mm) and log G* (the principal viscoelasticity index at 1 rad/s). Values are shown as mean ± SD for different treatment protocols.

Spinnability of CF Sputum treated with rhDNase and Gelsolin

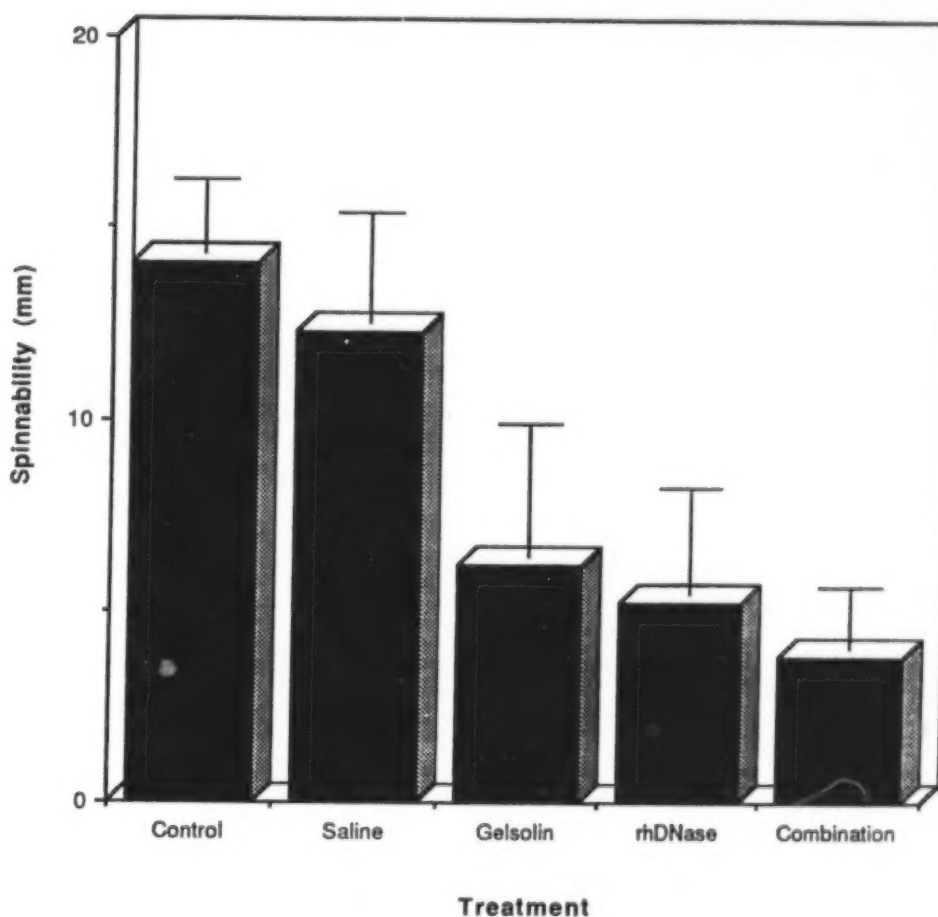


Figure IV-1

Individual and combined effects of rhDNase and Gelsolin on spinnability of CF sputum. Spinnability values (mean $\pm$ SD) for different treatment protocols and controls are shown in this figure.

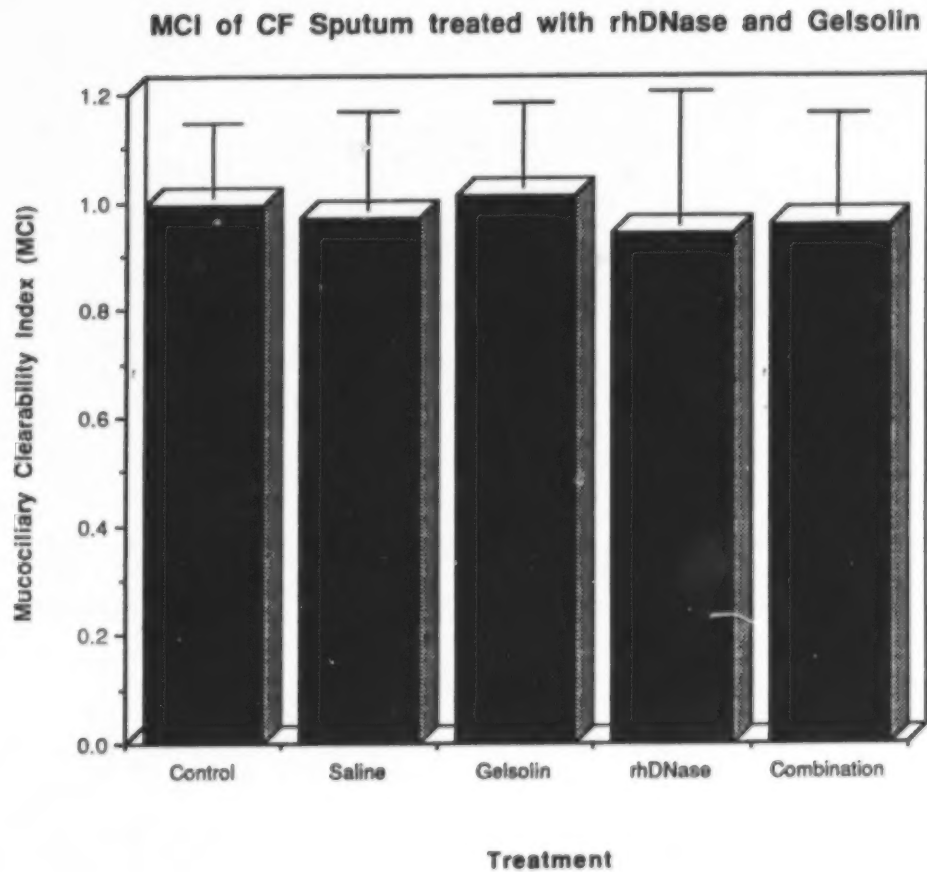


Figure IV-2

Individual and combined effects of rhDNase and Gelsolin on mucociliary clearability index (MCI) of CF sputum. MCI values (mean $\pm$ SD) for different treatment protocols and controls are shown in this figure.

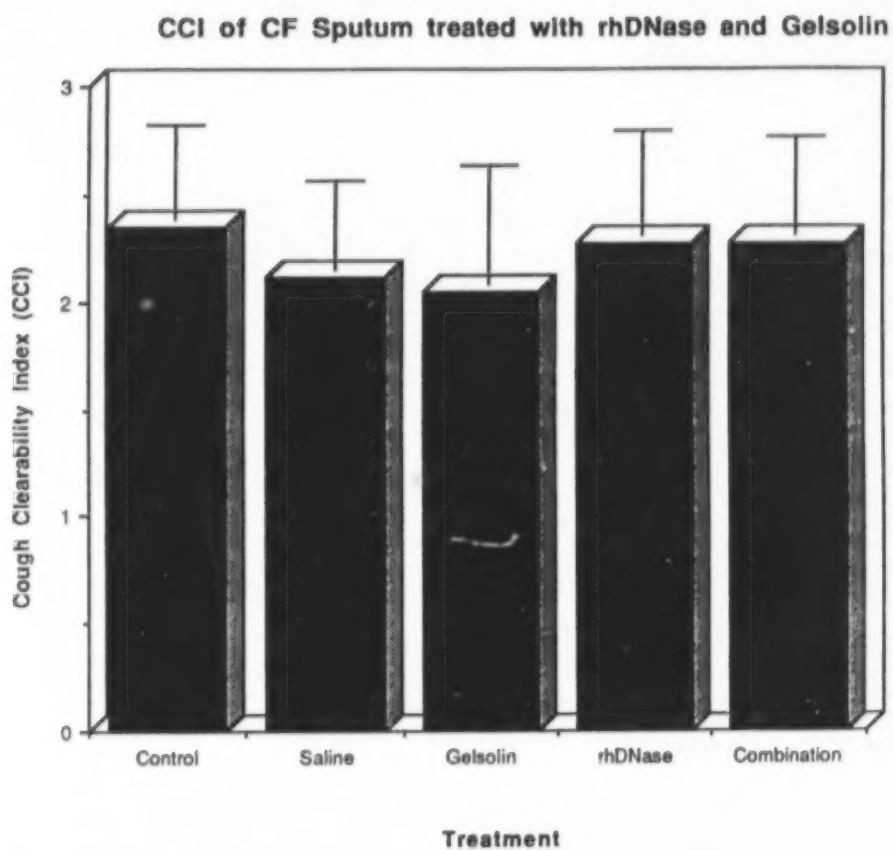


Figure IV-3

Individual and combined effects of rhDNase and Gelsolin on cough clearability index (CCI) of CF sputum. CCI values (mean $\pm$ SD) for different treatment protocols and controls are shown in this figure.

Chapter V

**REDUCTION IN VISCOELASTICITY OF CYSTIC FIBROSIS SPUTUM
IN VITRO WITH COMBINED TREATMENT BY NACYSTELYN AND
rhDNase**

INTRODUCTION

Respiratory dysfunction due the accumulation of purulent and mucoid sputum is characteristic of cystic fibrosis (CF) lung disease. To change the physical properties of the viscous and rigid mucus gel, the direct approach is mucolysis, the disruption of the mucous gel by altering the degree of crosslinking or interactions between the macromolecules that form the gel. This disruption can be achieved through pharmacological agents [referred to as Mucotropic Agents] such as Nacystelyn® (NAL) and recombinant human deoxyribonuclease I (rhDNase-Pulmozyme®). Previous studies have shown that the synergy of mucotropic agents demonstrate maximal effects on mucolysis (1).

NAL is composed of N-acetylcysteine (NAC) and L-lysine (Lys); its mucolytic action is based on the combined mucolytic activity of two different agents (2). Both NAC and NAL reduce the disulfide bonds (S-S) within the mucous gel to sulfhydryl functional group (-SH), reducing the viscosity of the mucus and enhancing expectoration through ciliary and/or cough mechanisms (3). Lys is believed to improve mucolytic action of NAC by increasing the pH of sputum. A recent *in vitro* study reported increased mucolytic activity with NAL in comparison with either NAC or Lys (4). *In vivo*, NAL also stimulated transepithelial chloride transport, thereby increasing the fluidity of the mucus, and thus improving its clearability (2).

Substantial amounts of deoxyribonucleic acid (DNA) of high molecular weight have been shown to be the leading cause of the tenacious and viscous nature of CF sputum (5-8). To combat DNA, aerosol administration of rhDNase has been used to reduce the viscosity of CF sputum, changing it from a nonflowing gel to a flowing liquid, thereby easing expectoration (7). A recent

clinical study has reported improved lung function after rhDNase treatment for 24 weeks (9).

In this investigation, the individual and combined effects of NAL and rhDNase were examined *in vitro* on the viscoelastic properties of CF sputum. To determine the viscoelastic properties, two variables were measured: (1) *spinnability* (in mm) - the capacity of mucus to form threads under traction; and (2) viscosity by *magnetic microrheometry* ($\log G^*$ at 1 rad/s) - the bulk viscosity and elasticity of microliter quantities of mucus.

MATERIALS AND METHODS

Subjects - Sputum samples were collected from eleven patients with CF by voluntary expectoration during a routine clinical visit. The patients (18 to 27 years, mean age 23 yrs) were all infected with *Pseudomonas aeruginosa* and treated with steroids, antibiotics, and bronchodilators as required. The patients had been exposed neither to rhDNase nor to NAL up to the time of sputum collection, and none were using any other type of mucolytic preparation. Approval to collect and use sputum for this *in vitro* analysis was obtained from the University of Alberta Research Ethics Board.

Study Design - Aliquots of each sputum sample (0.2 g) were subjected to four different treatment protocols: *i*) incubation with rhDNase [Pulmozyme®, Genentech, Inc.] in normal saline to achieve *ca.* 100 nM (2.5 µg/mL) final concentration; *ii*) incubation with NAL [Nacystelyn®, SMB & Galephar] in normal saline to achieve *ca.* 10 µM (3.09 µg/mL) final concentration; *iii*) no treatment (e.g. rhDNase, NAL), as a negative control; *iv*) incubation with 10% of normal saline, as a positive control to test the dilution effect. The samples in protocols *i*, *ii*, and *iv* were incubated at 37°C for 30 minutes.

To observe the combined effects of NAL and DNase, protocols *i* and *ii* were combined at half the concentration of each drug then incubated at 37°C for 30 minutes.

For each treatment protocol, spinnability, by filancemeter, and viscoelasticity ($\log G^*$), by magnetic microrheometer, were measured prior to any treatment (baseline), and then after 30 minutes of application of the treatment. Three spinnability readings per aliquot were taken, and the arithmetic mean of the three readings was calculated.

Rheological Measurements on CF Sputum - In this *in vitro* study, two techniques were used to measure the viscoelastic properties of CF sputum: the Filancemeter and the Magnetic Microrheometer. Spinnability is the thread forming ability of mucus under the influence of low speed elastic deformation, and was measured in mm by the filancemeter. The bulk viscosity and elasticity ($\log G^*$) was measured by means of a magnetic microrheometer. Specifically, from the $\log G^*$, two derivative parameters were calculated from *in vitro* relationships: mucociliary clearability index (MCI) and the cough clearability index (CCI). Based on model studies, both the MCI and the CCI can be used to predict the effect of mucus clearance, both by ciliary action and cough mechanism (10). Details of the filancemeter and the magnetic microrheometer are found in the Materials and Methods section of Chapter 4 of this thesis.

Statistical Analysis - Data from each protocol are presented as mean $\pm$ standard deviation (SD). To analyze the significance of changes in spinnability, MCI, and CCI after administration of rhDNase, and NAL, the sputum from each patient served as its own control. For each treatment, we determined the overall significance of the changes from baseline with the two-tailed paired t-test. The two-tailed paired t-test was also used to determine the differences between spinnability, MCI, and CCI after different types of treatment.

RESULTS

Spinnability Measurements

We observed that chemical mucolysis, by both singular and additive treatments of DNase and NAL, decreased spinnability of CF sputum *in vitro*. As indicated in Table V-1, 30 minutes of *in vitro* treatment of CF sputum with individual treatments of DNase and NAL resulted in a mean decrease in spinnability from baseline of 64% and 53%, respectively, whereas the mean decrease in spinnability after combined treatment of DNase and NAL was 78%. Similarly, when compared to their respective controls (0.9% saline), the combined treatment had greater effects on spinnability than either DNase or NAL at 30 minutes. NAL compared with saline gave a 45% decrease, DNase *versus* saline gave a 57% decrease, whereas combined treatment *versus* saline resulted in a 74% decrease.

Individual Effects of Mucolysis (Figure V-1) - The administration of NAL (10 μ M final concentration) over 30 minutes demonstrated a significant decrease in spinnability in comparison to incubation with normal saline (6.49 ± 1.94 vs. 11.73 ± 1.58 mm respectively, $p < 0.0001$). However, the administration of 100 nM of DNase decreased spinnability significantly more than either NAL or saline for 30 minutes (4.99 ± 2.20 mm). There was no significant difference in spinnability between administration of DNase and administration of NAL ($p = 0.22$) respectively over 30 minutes.

Combined Effects of Mucolysis (Figure V-1)- In comparison to treatment with normal saline, the combined treatment of DNase and NAL (half the above-mentioned concentration of each mucotropic agent) over a period of 30 minutes showed a significant decrease in spinnability ($p < 0.0001$). The combined

treatment also significantly reduced spinnability compared to NAL alone over the same period of time (3.01 ± 1.23 vs. 6.49 ± 1.94 mm respectively, $p = 0.002$) and to the administration of DNase alone over a period of 30 minutes (3.01 ± 1.23 vs. 4.99 ± 2.20 mm respectively, $p = 0.04$).

Viscoelasticity ($\log G^*$) and Clearance Indices (Table V-1)

The singular and the combined administration of NAL and DNase reduced the index of sputum rigidity, $\log G^*$ at 1 rad/s, insignificantly (1.82 ± 0.43 for NAL, 1.86 ± 0.22 for DNase, and 1.80 ± 0.48 for combination, respectively). However, the greatest reduction in $\log G^*$ at 1 rad/s was observed after saline treatment (1.76 ± 0.33). No significant changes in MCI and CCI resulted after singular and combined treatments of NAL and rhDNase, although mean MCI was highest for rhDNase treatment, and CCI was highest for the combination treatment.

DISCUSSION

CF sputum treated individually with either NAL or rhDNase demonstrated a significant decrease in spinnability when compared to CF sputum treated with saline. However, in comparison to singular treatments, the combined treatment of NAL and rhDNase, at half the concentration of each mucotropic agent, showed an even larger, statistically significant reduction in spinnability of CF sputum (Table V-1). This is an indicator of synergy in action between the two mucotropic agents.

The enzyme DNase has the ability to break down large concentrations of DNA, thus improving the viscoelastic properties of CF sputum, reducing sputum viscosity and enhancing expectoration (7). CF sputum treated with rhDNase demonstrated a reduction in spinnability similar to that reported in our previous studies (1,11-12). Zahm *et al.* (13) reported improvements in sputum transportability (by frog palate assay) with DNase at concentrations ranging from 0.2 to 20 $\mu\text{g/mL}$, except for a few samples at higher concentrations. They suggested that the ideal local concentration of DNase should be about 2 $\mu\text{g/mL}$, which is comparable to the final concentration used in this study.

NAL is a derivative of acetylcysteine, a thiol reducing agent which breaks disulfide bonds (14,15). NAC has been widely used to treat mucus clearance disorders (16). Previous studies have shown that NAL has greater mucolytic activity than NAC (4,17), and preliminary clinical trials with NAL have shown it to be effective in reducing the viscoelasticity of sputum in CF patients (18). Marriott *et al.*, using porcine gastric mucus (4), found significant mucolytic activity with NAL starting at 8 μM concentration, while App *et al.*, for sputum (17), reported significant activity at 10 μM , the same concentration that we used in the present study. Other thiol reducing agents, such as DTT or Mesna, may overliquify mucus (17), thereby making it unsuitable for clearance by ciliary action (19).

NAL might provide the appropriate balance of mucolytic action amongst thiol reducing agents.

In comparison to treatment with NAL, the mean reduction in spinnability due to rhDNase was greater, despite the lower concentration, although the difference was not significant. This tendency towards a greater sensitivity to DNase concurs with the results presented by Shah *et al.* (20), who reported that CF sputum samples were more responsive to DNase than to NAL, although in the latter study, the concentrations of both drugs were much higher than those used in the present investigation.

Both NAL and rhDNase demonstrated a significant reduction in sputum spinnability, but no significant changes were observed in $\log G^*$, or the derivative parameters, MCI and CCI. However, administration of saline resulted in a large reduction in $\log G^*$ at 1 rad/s, demonstrating a dilution effect. The change in the molecular weight of the CF sputum samples after administration of the mucolytic agents was thus demonstrated with the results of the filancemeter, and not by the results of the magnetic microrheometer.

These contrasting findings may be a consequence of the sensitivity of the diagnostic tools used. The reasons why spinnability by filancemeter could be a more sensitive indicator for this type of mucolysis (i.e. molecular weight reduction) than viscoelasticity by magnetic microrheometer are probably quite complex. The main reason is likely related to the molecular weight dependence of the two techniques. Viscosity (and probably viscoelasticity) classically exhibits a 3.4-power dependence on molecular weight (21). The power dependency for spinnability is less certain, but to the extent that it is related to the first normal stress difference, its power law dependency should be much higher, theoretically 6.8 power (22). This extra power dependency means that a mere 10% reduction in molecular weight would reduce spinnability to $(0.9)^{6.8}$ or

49% of control, which is comparable to the reduction in spinnability observed for NAL. At the same time, viscoelasticity should only decrease to $(0.9)^{3.4}$ or 70%. Because viscoelasticity ($\log G^*$) is usually measured on a logarithmic scale, this reduction would only amount to 0.16 log units, which is within the usual limit of detectability for the magnetic microrheometer (ca. 0.2 log units). Therefore, with relatively small changes in molecular weight of the CF sputum samples (resulting from occasional breakage of intramolecular bonds), alterations in spinnability measurements (using the filancemeter) could be readily observed, while changes in rigidity measurements (using the magnetic microrheometer) were not apparent.

The combined treatment of sputum with rhDNase and NAL (at half the concentration of each mucotropic agent) decreased spinnability significantly more than the singular treatments with either rhDNase or NAL. This synergistic behavior of NAL and rhDNase in greatly reducing the viscoelasticity of CF sputum may in part be due to cooperative rearrangements of the bonding and intermolecular interactions between neighbouring molecules. Reduction of the mucin disulfide bonds to sulfhydryl functional groups by NAL might make DNA more accessible for action by rhDNase. At the same time, cleavage of high molecular weight DNA by rhDNase, might assist in the reduction of sputum viscoelasticity by NAL. These favorable alterations in mucus rheology would predict an enhancement of expectoration and ultimately an improvement in the lung function of CF patients.

In conclusion, this *in vitro* study indicates that the combined treatment of mucus with NAL and rhDNase may indeed be a promising mucotropic modality to ease airflow limitation caused by the viscous airway secretions which are produced by CF patients. By combining rhDNase and NAL, mucolysis may not only be more appropriate for rheological change, but also more cost-effective in

treating disorders of impaired mucus clearance. Furthermore, by reducing the required dose of each agent, possible side-effects would be minimized. Additional studies with *in vivo* experiments and a larger patient population are required to confirm these findings and investigate issues regarding the safety and efficacy of the long-term administration of rhDNase and NAL in combination.

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Rheological effects of rhDNase and NAL treatment on CF sputum

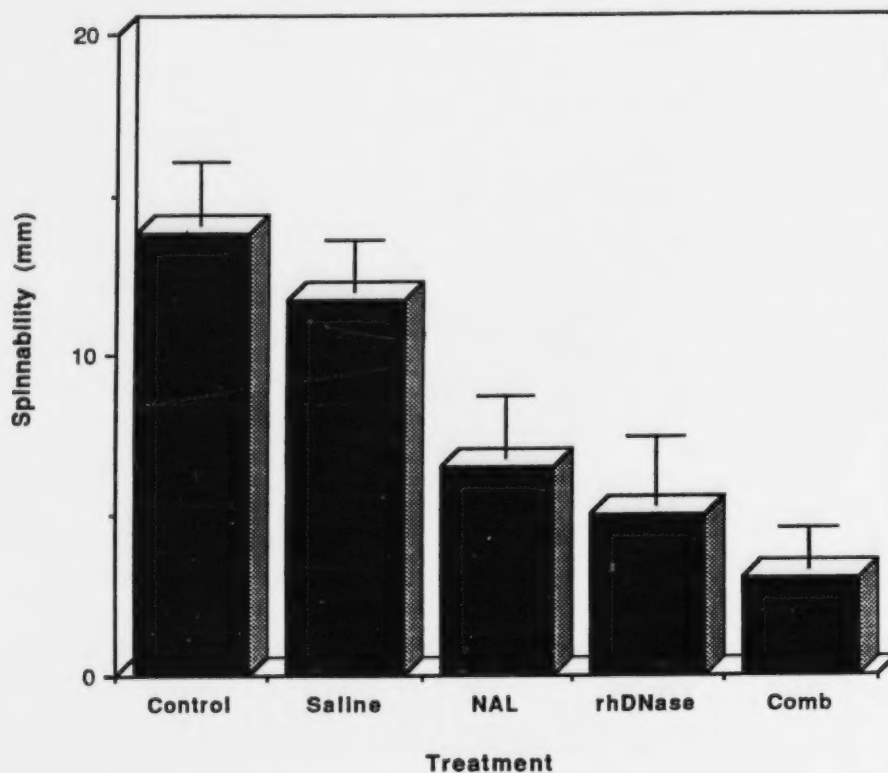
	BASELINE	SALINE	rhDNase	NAL	COMBINATION
Spinnability (mm)	13.86 ± 1.91	11.73 ± 1.58	4.99 ± 2.20 *§	6.49 ± 1.94 *†	3.01 ± 1.23 *
Viscoelasticity (log G* @ 1 rad/s)	1.90 ± 0.40	1.76 ± 0.33	1.86 ± 0.22	1.82 ± 0.43	1.80 ± 0.48
MCI	0.81 ± 0.09	0.86 ± 0.11	0.92 ± 0.12	0.83 ± 0.23	0.90 ± 0.08
CCI	1.30 ± 0.46	1.61 ± 0.34	1.70 ± 0.18	1.68 ± 0.43	1.78 ± 0.50

[*p < 0.0001 in comparison with saline by paired t-test; §p = 0.04 compared with combination;

†p = 0.002 compared with combination]

Table V-1

Singular and combined effects of rhDNase and Nacystelyn (NAL) on spinnability (mm), log G* (the principal viscoelasticity index at 1 rad/s), mucociliary clearability index (MCI), and cough clearability index (CCI). Values are shown as mean ± SD for different treatment protocols.

Effects of NAL and rhDNase on Spinnability of CF sputum**Figure V-1**

Individual and combined effects of rhDNase and Nacystelyn (NAL) on spinnability of CF sputum. Spinnability values (mean $\pm$ SD) for different treatment protocols and controls are shown in this figure.

Chapter VI

**COMBINED EFFECTS OF HYPERTONIC SALINE AND rhDNase ON
CYSTIC FIBROSIS SPUTUM *IN VITRO***

INTRODUCTION

Chronic pulmonary infection is the major cause of morbidity and mortality in cystic fibrosis (CF) lung disease. Most adults have lungs which are colonized with *Pseudomonas aeruginosa*; the most frequent cause of death is respiratory failure due to bronchopulmonary sepsis (1). Accumulation of infected viscous secretions is the major cause of lung damage in CF. Therefore, current treatment of CF lung disease includes strategies aimed at changing the physical properties of pulmonary secretions to improve mucociliary clearability in CF patients.

In vitro, hypertonic saline (HS) acts by changing the hydrogen and ionic bonding of the mucous gel while, *in vivo* it acts as an expectorant promoting mucus secretion (2). The interaction of HS with a mucosal surface results in both extraction of water from the tissue and the transfer of ions into the tissue until the osmotic pressure of the solution becomes isotonic. The extra volume of water that is withdrawn from the tissue may result in either blockage of the small airways, or dilution of the mucous gel reducing its viscosity (2). For the purpose of this study, the effects of HS as a mucotropic agent were observed.

The two macromolecules, DNA and mucous glycoproteins, have been reported to be the major contributors of the physical properties of CF airway secretions (3). Secretions from CF patients have DNA content of up to 10.2% of the dry weight (4); DNA has been reported to accumulate at an average concentration of 5.9 mg/mL (5). A recent study reported administration of recombinant human deoxyribonuclease I (rhDNase - Pulmozyme®) to decrease the concentration of high molecular weight DNA molecules, reducing the abnormal viscoelastic properties of CF sputum and improving airway mucus clearance (6).

This *in vitro* study examined the effects of HS in combination with

rhDNase on the viscosity and clearability of CF sputum. The individual effects of HS were observed first, followed by determining the combined effects of HS and rhDNase, and of saline (0.9% NaCl) and rhDNase.

MATERIALS AND METHODS

Subjects - Sputum samples were collected from six patients with CF by voluntary expectoration during a routine clinical visit. The patients (18 to 37 years, mean age 27 yrs) were all infected with *Pseudomonas aeruginosa* and treated with steroids, antibiotics, and bronchodilators as required. None of the patients had been exposed to either rhDNase or HS aerosol up to the time of sputum collection, and none were using any other type of mucolytic preparation. Approval to collect and use sputum for this *in vitro* analysis was obtained from the University of Alberta Research Ethics Board.

Study Design - Aliquots of each sputum sample (0.2 to 0.4 g) were subjected to four different treatment protocols: *i*) incubation with 3% HS; *ii*) negative control, without application of any treatment (e.g. rhDNase, HS). *iii*) positive control, to test the dilution effect, treated with ten percent of normal saline (NS). The samples in protocols *i* and *iii* were incubated at 37°C for 30 minutes.

To observe combined effects of HS or NS with rhDNase, protocols *i* and *iii* were combined with ca. 100 nM concentration of rhDNase [Pulmozyme®, Genentech, Inc.] then incubated at 37°C for 30 minutes.

For each treatment protocol, spinnability and log G^* were measured and mucociliary clearability index (MCI), and cough clearability index (CCI) were calculated prior to any treatment (baseline), and then after 30 minutes of application of the treatment. Three spinnability readings per aliquot were taken, and the arithmetic mean of the three readings was calculated.

Rheological Measurements on CF Sputum - In this *in vitro* study, two techniques were used to measure the viscoelastic properties of CF sputum: the Filancemeter and the Magnetic Microrheometer. Spinnability is the thread

forming ability of mucus under the influence of low speed elastic deformation, and was measured in mm by the filancemeter. The bulk viscosity and elasticity ($\log G^*$) was measured by means of a magnetic microrheometer. Specifically, from the $\log G^*$, two derivative parameters were calculated from *in vitro* relationships: MCI and the CCI. Based on model studies, both the MCI and the CCI can be used to predict the effect of mucus clearance, both by ciliary action and cough mechanism (7). Details of the filancemeter and the magnetic microrheometer are found in the Materials and Methods section of Chapter 4 of this thesis.

Statistical Analysis - Data from each protocol are presented as mean $\pm$ standard deviation (SD). To analyze the significance of changes in spinnability, MCI, and CCI after administration of NS, rhDNase, and HS, the sputum from each patient served as its own control. For each treatment, we determined the overall significance of the changes from baseline with the two-tailed paired t-test. The two-tailed paired t-test was also used to determine the difference between spinnability, MCI, and CCI after different types of treatment.

RESULTS

Spinnability Measurements

Compared to baseline, 30 minutes of single treatments with NS and HS resulted in a mean decrease in spinnability of CF sputum from baseline of 16% and 26%, respectively. The mean decrease in spinnability for the combined treatment of NS and DNase was 37%, while after combined treatment of HS and DNase the decrease was 40%. When compared to 0.9% NS as a control (i.e. at 30 minutes), HS vs. NS gave a 12% decrease, and the combination of NS and DNase treatment vs. NS gave a 25% decrease, whereas the combination of HS and DNase vs. NS treatment resulted in a 29% decrease. The combined *in vitro* treatment of rhDNase and HS on CF sputum decreased the spinnability (similar to the decrease demonstrated after *in vitro* treatment with NS [0.9% NaCl] on CF sputum) (1). These results on spinnability are presented in Table VI-1.

Individual Effects of Mucolysis (Figure VI-1)- Administration of HS over 30 minutes resulted a small decrease in spinnability in comparison to incubation with NS (10.7 ± 0.61 vs. 12.18 ± 0.79 mm respectively, $p = 0.0085$). There was no significant difference in spinnability between administration of NS and the control sample, which had no treatment applied (12.18 ± 0.79 vs. 14.43 ± 1.17 mm, respectively) over 30 minutes.

Combined Effects of Mucolysis (Figure VI-1) - After a period of 30 minutes, the combined treatment of DNase and HS decreased spinnability more than administration of HS alone over the same period of time (8.67 ± 1.03 vs. 10.7 ± 0.61 mm respectively, $p = 0.0009$). The combined treatment of DNase and NS also decreased spinnability significantly more than administration of HS alone

over a period of 30 minutes (9.10 ± 1.04 vs. 10.7 ± 0.61 mm respectively, $p = 0.002$).

Rheology and Clearance Indices

HS demonstrated improvements in the sputum rigidity index ($\log G^*$ at 1 rad/s) compared to treatment with NS (1.41 ± 0.49 for HS vs. 1.76 ± 0.25 for NS) [Table VI-1]. Combined treatment with HS and DNase also showed modest improvements compared to CF sputum treated with NS and DNase (1.64 ± 0.34 for HS + DNase vs. 1.79 ± 0.29 for NS + DNase respectively). As indicated in Figures VI-2 and VI-3, HS also demonstrated significant changes in both the MCI and the CCI of CF sputum in comparison to combined treatment with NS and DNase (1.08 ± 0.12 for HS vs. 1.00 ± 0.11 for NS + DNase, $p = 0.0006$ for MCI, and 2.11 ± 0.34 for HS vs. 1.63 ± 0.26 for NS + DNase, $p = 0.04$ for CCI). A significant change was also observed in the CCI between singular HS treatment and combined treatment of HS and DNase (2.11 ± 0.34 for HS vs. 2.45 ± 1.76 for HS + DNase, $p = 0.04$).

DISCUSSION

Addition of HS to CF sputum demonstrated a small reduction in spinnability compared to treatment with NS. Treatment of CF sputum with rhDNase, as in our previous studies (8-10), resulted in a significant decrease in spinnability. Although the combined treatment of HS and DNase showed a greater reduction in spinnability than either treatment by itself, the reduction was less than additive. Thus, as opposed to Gelsolin (9) and Nacystelyn (10), in this study, we saw no evidence for synergy between DNase and HS.

Previous *in vitro* studies report HS to be more effective in reducing mucoid sputum viscosity in comparison to water (11,12). An increase in mucociliary clearability was also demonstrated in this study. These *in vitro* results correlate with work done by Pavia *et al.* (13), who observed enhanced mucociliary clearance rates in patients with chronic bronchitis after inhalation with 1.21 M NaCl. Robinson *et al.* (14), in adult CF patients, used the same concentration (7% NaCl, 1.20 M) and observed an increased rate of clearance of tracer particles from the lung when compared with either amiloride aerosol or cough. After observing the results of our study and work done by others (13,14), it would seem that HS solutions stimulate mucociliary transport in both chronic bronchitis and CF patients.

The sputum samples in the present study were treated with 3% NaCl (0.512 mM) [1 part to 10 parts of sputum]. The initial sputum NaCl content was not measured, but based on previous literature reports, it should have been about 88 mM (mean Na^+ =101 mM, mean Cl^- =76 mM). Thus, the final concentration of NaCl in the sputum treated with 3% NaCl was about 126 mM. This is less than serum levels and less than that reported for laryngectomized patients by Potter *et al.* (165 mM) (15).

The increase in NaCl in this *in vitro* treatment was *ca.* 38 mM, resulting in a modest decrease in spinnability compared with NS, as well as a decrease in

viscoelasticity by magnetic microrheometry. The increase in NaCl content was comparable to that reported by Tomkiewicz *et al.* (16) for long-term amiloride treatment of CF patients, where the mean NaCl content went from 95 to 121 mM, and was associated with a significant decrease in viscoelasticity despite no detectable change in sputum water content. It was suggested at the time that CF sputum rheology might be particularly susceptible to small changes in ion content (17), and the results of the current study would seem to confirm this.

The combined treatments of DNase and HS and DNase and NS showed a greater reduction in spinnability in comparison to individual treatments of HS alone. It can thus be concluded from these results that the additive effects of both HS and DNase and DNase and NS in reducing the sputum viscoelasticity is largely due to DNase. Treatment with rhDNase has been reported to cleave neutrophil-derived DNA present in CF infected lungs, reducing the adhesiveness and viscoelasticity of CF sputum (5). By separating the DNA molecules from the mucoprotein, it was expected that HS would make the DNA more susceptible to enzyme digestion (18). However, this was not the case as indicated by the results. The reason why this combination of HS and DNase is less than additive in its effect on spinnability may be due to the reduction in activity of DNase at high salt concentrations (S. Shak personal communication), given the fact that DNase and HS were combined prior to incubating the sputum.

In the previous papers (Chapters 4 and 5 of this thesis), the change in the molecular weight of the CF sputum samples after mucolytic agents were administered was demonstrated with the results of the filancemeter, but not by the results of the magnetic microrheometer. The reasons why spinnability by filancemeter appears to be a more sensitive indicator for this type of mucolysis (i.e. molecular weight reduction) than viscoelasticity by magnetic

microrheometer are probably quite complex. The main reason is likely related to the molecular weight dependence of the two techniques. Viscosity (and probably viscoelasticity) classically exhibits a 3.4-power dependence on molecular weight (19). The power dependency for spinnability is less certain, but to the extent that it is related to the first normal stress difference, its power law dependency should be much higher, theoretically 6.8 power (20). This extra power dependency means that a mere 10% reduction in molecular weight would reduce spinnability to $(0.9)^{6.8}$ or 49% of control, which is comparable to the reduction in spinnability observed for rhDNase treatment. At the same time, viscoelasticity should only decrease to $(0.9)^{3.4}$ or 70%. On the logarithmic scale used for viscoelasticity ($\log G^*$), this reduction would only amount to 0.16 log units, which is within the usual limit of detectability for magnetic microrheometer (ca. 0.2 log units). With hypertonic saline treatment, it is assumed that there is no cleavage of intramolecular bonds and no reduction in molecular weight. However, the treatment (shielding of ionic charge) should produce substantial reductions in intermolecular interaction and macromolecular conformation, both of which will reduce the degree of entanglement coupling. For this type of alteration, viscoelasticity and spinnability changes should become comparable (21).

Hence, this *in vitro* study demonstrated that combined treatments of both HS and DNase and DNase and NS: (i) decreases the spinnability and $\log G^*$ (the principal viscoelasticity index), and (ii) increases the cough clearability index moderately more than individual treatments with either HS or saline. These results of combined treatment with HS and rhDNase seem encouraging since they demonstrate a change in the physical property of CF sputum from a rigid and purulent gel to a free flowing liquid. Because of the possibility that co-administration of HS with rhDNase might reduce the effectiveness of rhDNase,

alternate or consecutive treatments with these two forms of mucotropic agents should be considered. Further studies are required to confirm these findings and to carry out similar experiments with different ratios of HS/rhDNase.

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Rheological effects of rhDNase and Hypertonic Saline treatment on CF sputum

	SALINE	HS	NS+DNase	HS+DNase
Spinnability (mm)	12.2 $\pm$ 0.79	10.7 $\pm$ 0.61	9.1 $\pm$ 1.04 *	8.67 $\pm$ 1.03§
Viscoelasticity (log G* @ 1 rad/sec)	1.76 $\pm$ 0.25	1.41 $\pm$ 0.49	1.79 $\pm$ 0.29	1.64 $\pm$ 0.34

[*p < 0.002 in comparison with HS by paired t-test; §p < 0.0009 compared with combination]

Table VI-1

Singular and combined effects of rhDNase, Hypertonic Saline (HS), and Normal Saline (NS) on spinnability (mm) and log G* (the principal viscoelasticity index at 1 rad/s). Values are shown as mean $\pm$ SD for different treatment protocols.

Effects of Hypertonic Saline & rhDNase on Spinnability of CF sputum

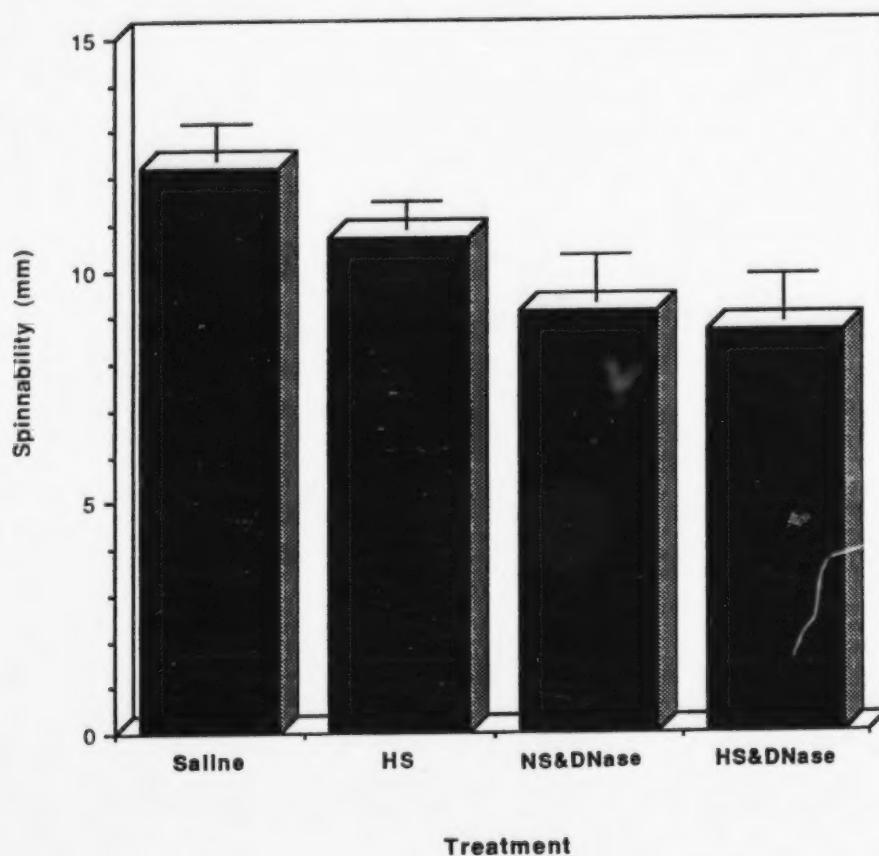
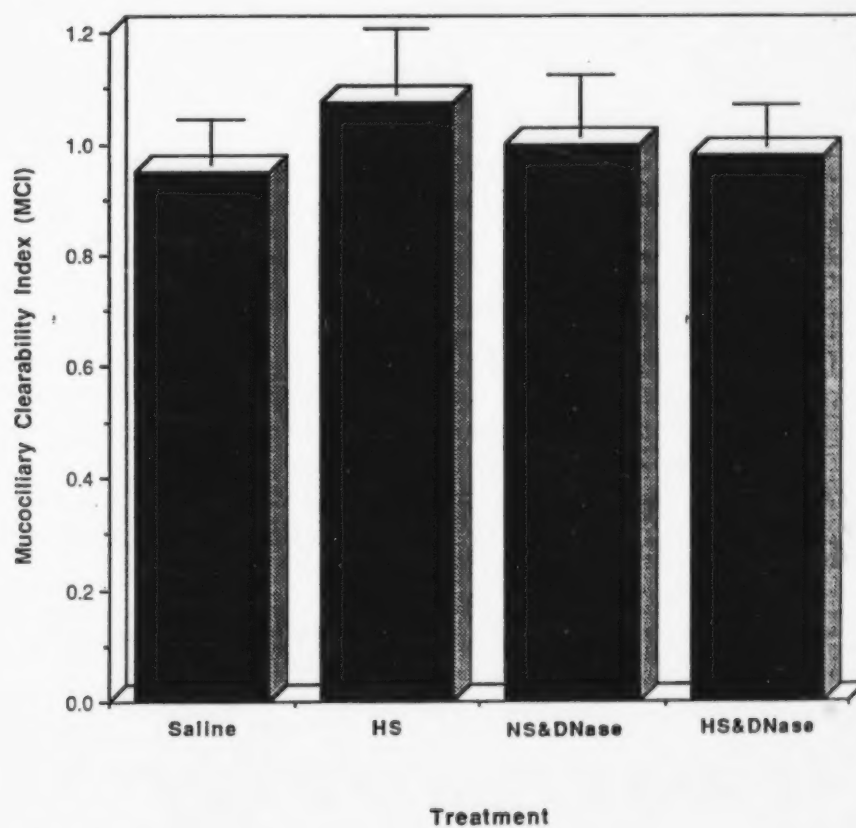
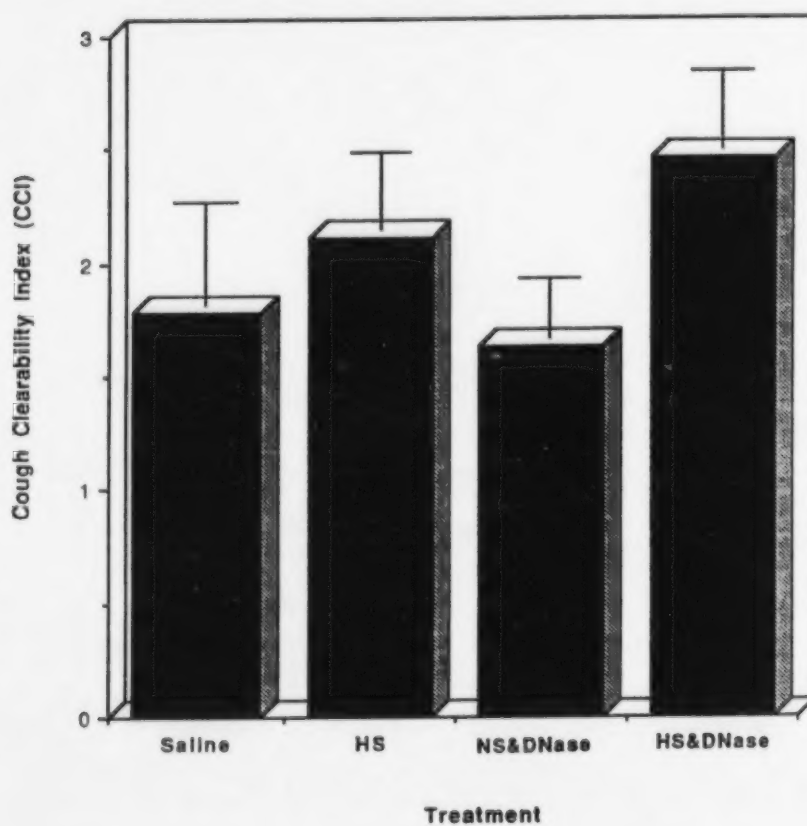


Figure VI-1

Individual and combined effects of rhDNase, Hypertonic Saline (HS), and Normal Saline (NS) on spinnability of CF sputum. Spinnability values (mean $\pm$ SD) for different treatment protocols and controls are shown in this figure.

Effects of Hypertonic Saline and rhDNase on MCI of CF sputum**Figure VI-2**

Individual and combined effects of rhDNase, Hypertonic Saline (HS), and Normal Saline (NS) on mucociliary clearability index (MCI) of CF sputum. MCI values (mean $\pm$ SD) for different treatment protocols and controls are shown in this figure.

Effects of Hypertonic Saline & rhDNase on CCI of CF sputum**Figure VI-3**

Individual and combined effects of rhDNase, Hypertonic Saline (HS), and Normal Saline (NS) on cough clearability index (CCI) of CF sputum. CCI values (mean $\pm$ SD) for different treatment protocols and controls are shown in this figure.

Chapter VII
GENERAL DISCUSSION AND CONCLUSIONS

The three-dimensional structure of the mucous gel is dependent on the following bonds and crosslinkages: 1) *covalent bonds*, 2) *ionic bonds*, 3) *hydrogen bonds*, 4) *van der Waals forces*, and 5) *physical entanglements*. To determine the different approaches to mucolysis, five representative mucotropic modalities were examined: (1) recombinant human deoxyribonuclease I (rhDNase), (2) Gelsolin, (3) Nacystelyn [NAL], (4) Hypertonic Saline [HS], and (5) Oscillation. The basis for comparison with the four other mucotropic agents was rhDNase (Pulmozyme®), a licensed mucolytic.

The first *in vitro* study of this series of experiments demonstrated that the additive treatment of DNase and oscillation decreased spinnability of CF sputum significantly more than with other types of singular or additive treatments [e.g. oscillations alone or saline and oscillation]. The significant decrease in spinnability of CF sputum treated with DNase may be related to its mechanism of action. DNase cleaves high molecular weight DNA [which causes the sputum to be viscous and tenacious] to low molecular weight DNA, thereby reducing the viscoelasticity of CF sputum (1). The decrease in spinnability of oscillated CF sputum may be explained by the reduction of polymer size induced through lysis of macromolecular backbone linkages. Oscillations mechanically disrupt the complex mucous gel structure, analogous to the action of rhDNase to reduce the molecular length of DNA in order to decrease the spinnability of CF sputum.

In physical mucolysis, physical interaction disrupts and disentangles mucus macromolecules. Yet chemical mucolysis, carried out by classical mucolytic agents such rhDNase, alters the bonding and intermolecular interactions between neighbouring macromolecules. This explains the increase in spinnability of the negative control sample, with no treatment applied, [as

shown in Figure III-1, chapter III of this thesis] which may have been caused by a slow drying effect followed by increased molecular interaction.

The second *in vitro* study dealt with the synergistic effects of gelsolin and rhDNase. The combined treatment of rhDNase and gelsolin decreased spinnability significantly more than the individual treatments of rhDNase or gelsolin. Gelsolin is an actin binding protein which rapidly severs noncovalent bonds between monomers within a filament (2), and rhDNase, in addition to cleaving high molecular weight DNA, has been reported to bind and slowly depolymerize actin filaments (3,4); therefore both rhDNase and gelsolin were combined *in vitro*. By breaking both the viscoelastic actin filaments and the DNA molecules, gelsolin and rhDNase were able to reduce the viscosity of CF sputum.

The third *in vitro* study dealt with the synergistic effects of NAL and rhDNase. By reducing the disulfide bond (S-S) to a sulfhydryl functional group (-SH), NAL was successful in reducing the elasticity and viscosity of mucus (5). The enzyme rhDNase acts by cleaving the DNA molecules, reducing the degree of viscosity of CF sputum (1). The combined treatment of rhDNase and NAL decreased spinnability significantly more than the singular treatments of either mucotropic agent. This reduced viscoelasticity triggered by the use of NAL and rhDNase is a result of their altering the intermolecular bonds between neighbouring molecules within the mucus. Therefore, the combination of both mucotropic agents significantly decreases the viscosity of CF sputum, thereby enhancing expectoration and improving lung function.

The fourth study looked at the *in vitro* additive effects of HS and rhDNase on CF sputum. The combination of rhDNase and HS decreased spinnability and increased cough clearability index [CCI] more than singular treatments of either one. Previous *in vitro* studies report HS to be more effective in reducing

mucoid sputum viscosity in comparison to water (6). By separating the DNA molecules from the mucoprotein, HS makes the DNA more susceptible to enzyme digestion (7). After observing the results of our study and work done by others (8,9), it would seem as though HS solutions stimulate mucociliary transport not only in CF patients but also in patients suffering from other disorders characterized by impaired mucus transport (such as chronic bronchitis).

In conclusion, as demonstrated above, a variety of techniques are involved in reducing the viscoelasticity of the mucous gel. Several different combinations were examined for this thesis: (1) rhDNase and oscillations, (2) rhDNase and gelsolin, (3) rhDNase and NAL, and (4) rhDNase and HS. Our basis for comparison was rhDNase, a licensed mucolytic agent, approved by Health and Welfare Canada and the Food and Drug Administration of the United States of America.

Overall the study demonstrated significant results in many areas, nonetheless some limitations were also observed in this study:

(1) Mucus analysis, measuring the spinnability and rheology of sputum, is a limiting technique. Although it is very appropriate, its limitation is that the diagnostic tools (such as the Filancemeter and the Magnetic Microrheometer) are not available throughout the world. Only five laboratories globally have the magnetic microrheometer and very few laboratories have a filancemeter; fortunately, the Pulmonary Research Group at the University of Alberta has both these instruments.

(2) Sputum can generally be collected from adolescents and adults who are suffering from an obstructive pulmonary disease like CF. Younger people (below the age of 12 years) have difficulties in expectorating sputum,

swallowing it instead, while healthy individuals are not capable of producing sputum, except by invasive means.

(3) There are some technical difficulties as well. Due to the small samples of mucus, it was not possible to measure the cough clearability using the cough clearability machine. Also, small samples of mucus are more vulnerable to drying and degradation.

(4) The sample size of the study population was also a limiting factor. Further studies are required using a larger population of both CF patients and patients suffering from other pulmonary disorders (e.g. asthma, chronic bronchitis).

But, in summary, the study has shown positive results in the following areas:

(1) The thesis has helped to further my understanding of the molecular nature of crosslinking and bonding in mucin gels by examining the novel types of mucolysis in terms of their mechanism of action on the structure and biochemistry of the mucous gel.

(2) Better insight has been achieved regarding the molecular action of different mucotropic agents on two different study populations – infected CF sputum and healthy tracheal dog mucus.

(3) It has been demonstrated that the filancemeter and magnetic microrheometer are useful diagnostic tools to observe the mechanism of action of mucotropic agents. Spinnability measures the viscoelasticity of sputum and rheology measures the bulk viscosity and elasticity of microliter quantities of sputum. A decrease in viscosity and elasticity of sputum will increase the clearability of mucus, which should lead to improved lung volumes and expiratory flow rates in patients with abnormalities in mucus clearance.

(4) The most important point is that the additive treatment with mucotropic agents, in combination with diagnostic tools used for mucus analysis, seems to

be the best direct approach to treating disorders of mucus clearance in CF patients. As the best mucolytic therapy varies from patient to patient, these results will be able to justify the development of a laboratory mucolytic sensitivity assay. This assay will be able to determine the right combination of mucotropic agents for each individual CF patient before initiating treatment.

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